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13. ABSTRACT ***(Maximum 200 Words)***

Vitamin D and its analogs are growth-suppressing and cell-differentiating but also have high calcemic activity. We identified a new synthetic vitamin D analog: $1\alpha(OH)D_5$. In malignant breast cells, it induced expression of differentiation markers, namely $\alpha 2$ integrin intracellular lipid and casein. Human breast carcinoma cells treated *in vitro* with $1\alpha(OH)D_5$ failed to form tumor when injected into athymic mice. *In vivo*, in athymic mice, dietary supplementation of $1\alpha(OH)D_5$ inhibited tumor growth and appeared to induce differentiation of transplanted human breast carcinoma cells. Animals receiving dietary $1\alpha(OH)D_5$ showed no serious deleterious effects except slight but significant increases in serum calcium. Serum calcium levels declined to that of control baseline level within one week of discontinuation of the treatment. We conjugated $1\alpha(OH)D_5$ with Her-2 antibody and evaluated its therapeutic efficacy in an experimental animal model. The $1\alpha(OH)D_5$-Her-2 conjugate inhibited the in vivo growth of breast carcinoma cells transplanted into athymic mice. The immunoconjugate was more effective than either agent administered alone in inhibiting growth of breast cancer cells. Animals receiving immunoconjugate showed no signs of toxicity or hypercalcemia.

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Introduction

1.Title of the Proposal: Breast carcinoma cell targeted therapy by novel vitamin D analog

2.PI Name: Rajeshwari R. Mehta, Ph.D.

3.Five Key Words in the Proposal: Cell differentiation, Vitamin D analog, chemotherapy, immunoconjugate, targeted therapy.

4.Abstract:

Vitamin D and its analog have growth-suppressing and cell-differentiating actions in various neoplastic cell types. However, their use as therapeutic or chemopreventive agents is hindered due to their high calcemic activity. We recently studied the effects on breast carcinoma cells of a new synthetic vitamin D analog: $1\alpha(OH)D_5$. In malignant breast cells, it induced expression of differentiation markers, namely $\alpha 2$ integrin intracellular lipid and casein. Human breast carcinoma cells treated *in vitro* with $1\alpha(OH)D_5$ failed to form tumor when injected into athymic mice. *In vivo*, in athymic mice, dietary supplementation of $1\alpha(OH)D_5$ inhibited tumor growth and appeared to induce differentiation of transplanted human breast carcinoma cells. Animals receiving dietary $1\alpha(OH)D_5$ showed no serious deleterious effects, even though slight but significant increases in serum calcium level were noticed. The effect of $1\alpha(OH)D_5$ was reversible, as serum calcium levels declined to that of control baseline level within one week of discontinuation of the treatment. These results suggested that $1\alpha(OH)D_5$ is a potent inducer of cell differentiation. We further explored cell-differentiating action. We targeted breast cancer cells with $1\alpha(OH)D_5$. We conjugated $1\alpha(OH)D_5$ with Her-2 antibody and evaluated its therapeutic efficacy in an experimental animal model. The $1\alpha(OH)D_5$-cerbB2 conjugate, even if administered at a low dose, will be more effective than $1\alpha(OH)D_5$ or c-erbB2 administered alone. We will also evaluate the therapeutic potential of $1\alpha(OH)D_5$ as a dietary supplement. We hypothesize that $1\alpha(OH)D_5$ could induce differentiation breast cancer cells, render them non-aggressive, and could alter their metastatic potential. The $1\alpha(OH)D_5$-Her-2 conjugate inhibited the in vivo growth of breast carcinoma cells transplanted into athymic mice. The immunoconjugate was more effective than either agent administered alone in inhibiting growth of breast cancer cells. Animals receiving immunoconjugate showed no signs of toxicity or hypercalcemia, as evident from the serum calcium analysis.

Based on the results obtained in this study, we applied for the CTR proposal which was awarded in 1999.

Body

Key Research Accomplishments

In recent years, several natural and synthetic agents, especially those with antiproliferative and differentiating properties, have been the primary focus of therapeutic and chemopreventive research. A synthetic analog of vitamin A, N-[4-hydroxyphenyl retinamide (HPR), is recognized as a chemopreventive agent for breast carcinoma in experimental animals. In addition to vitamin A, Vitamin D has also shown promising results. Vitamin D is classified as a hormone within a steroid hormone family.[5] It is a secosteroid that is biologically inert until hydroxylated on the carbon 25 position in the liver to form 25-hydroxyvitamin D, which is further metabolized to 1α,25 dihydroxy vitamin D_3 ($1\alpha D_3$). When it is no longer needed, the hormone gets metabolized to an inactive form (24-hydroxyvitamin D) and excreted from the body.[6] In addition to its function in maintaining blood calcium level and mobilizing calcium from bone, $1\alpha(OH)D_3$ has growth-suppressive and cell-differentiating actions in many malignant cell types.[7,8]

One major factor limiting successful use of vitamin D or $1\alpha D_3$ in cancer prevention or therapy is its calcemic activity. The concentration needed to cause reduced growth of neoplastic cells would cause hypercalcemia and death. Therefore, in recent years, attention has been directed to developing analogs that preserve vitamin D's growth suppressive activity but reduce its calcemic activity.[9] In experimental systems, addition of vitamin D analogs to adriamycin or tamoxifen treatment has shown enhanced growth inhibitory action of drugs.[10,11] We recently evaluated a novel vitamin D analog, $1\alpha(OH)D_5$, as a potential antiproliferative or cell-differentiating agent for breast cancer cells. This analog was synthesized by Dr. Robert M. Moriarty, Professor, in the Department of Chemistry, University of Illinois at Chicago. Table 1 summarizes the results obtained previously in our laboratory. $1\alpha(OH)D_5$ is nontoxic in athymic mice.

Table 1

***In vivo* and *in vitro* effects of $1\alpha(OH)D_5$ on premalignant and malignant breast cells (Preliminary results from our laboratory)**

Evaluation	Animal model used	Optimal Effective dose	Results
Chemopreventive effect	Mouse mammary gland organ culture[12] system, effect on DMBA-induced preneoplastic lesions (*In vitro*)	1 μM	$1\alpha(OH)D_5$ inhibited DMBA-induced premalignant lesion formation *in vitro* [12]
Growth inhibitory effect	Human breast cancer cell lines, *UISO-BCA-1, *BCA-2, *BCA-4, T47D, MCF-7 (*In vitro*).	1 μM (10 days exposure)	$1\alpha(OH)D_5$ had dose related growth inhibitory effect on BCA-4, MCF-7, T47 D cell lines, irrespective of their estrogen and progesterone receptor status[13]
Cell differentiating effect	Human breast cancer cell lines, UISO-BCA-1, BCA-4 and MCF-7 *In Vitro*)	1 μM (7 days exposure)	It induced expression of various proteins associated with cell differentiation, namely nm23, ICAM-1, casein[13]
Other effects on various other biomarkers	Human breast cancer cell lines, UISO-BCA-4 (*In vitro*)	1 μM, 7 days exposure	Histological changes, induction of TGFB1, VDR[13] Downregulation of UPA, UPAR, TGFB3, EGFR,BCL-2
Effect on *in vivo* Tumorigenicity	Human breast carcinoma cell line BCA-4 (In athymic mice)	8 ng/animal 3 times weekly for 2 months, s.c. injection	Complete inhibition of tumor growth, originally injected cells appeared differentiated histologically
Toxicity	in mice (*In vivo*)	At 400 ng/animal, 3 times weekly, 21 days treatment	No apparent toxicity, (non-calcemic activity) Expts. are in progress at higher dose

*These cell lines are established by the PI and are characterized in detail.[14,15]

Determine dietary optimal effective dose with no toxicity (calcemic activity and other toxic effects) in athymic mice. We have tested the stability of the analog after

mixing it in the diet and keeping it at room temperature; the compound is stable for several days at room temperature.

Original Hypothesis

Vitamin D and its analogs have growth-suppressing and cell-differentiating actions in various neoplastic cell types. However, their use as therapeutic or cancer preventive agents is hindered due to their high calcemic activity. We have recently studied the effects of a new synthetic vitamin D analog $1\alpha(OH)D_5$ in breast carcinoma cells. This newly synthesized analog appears to have no significant calcemic activity. In experimental systems, $1\alpha(OH)D_5$ inhibited the development of premalignant lesions in DMBA-exposed mammary gland explants. In malignant cells, it induced expression of various markers (namely I-CAM, casein, and nm23) associated with breast cell differentiation. Also, altered phenotypic changes were associated with induction of vitamin D receptor (VDR) and $TGF\beta_1$ protein. In women, tumors showing overexpression of nm23, ICAM, and e-cadherin are generally noninvasive. In the present study, we hypothesize that $1\alpha(OH)D_5$ treatment could induce breast cancer cell differentiation, render them non-aggressive, and alter their tumorigenicity and metastatic potential. If vitamin D analog proves to induce functional and biological differentiation in breast carcinoma cells, it will be of great value as a chemopreventive agent, particularly in women with premalignant lesions and at a high risk of developing aggressive tumor. $1\alpha(OH)D_5$ could be easily given as dietary supplement. Alternatively, it could be administered at low concentration as an immunoconjugate with c-erbB2 antibody and specifically targeted for breast carcinoma cells, without any effect on normal cells.

Technical Objectives proposed

In the present study, we evaluate the potential therapeutic and antimetastatic properties of $1\alpha(OH)D_5$. Original specific aims proposed are to:

1) Determine effects of $1\alpha(OH)D_5$, a synthetic vitamin D analog, on morphological or phenotypic, functional, and biological characteristics of malignant cells.

2) Evaluate therapeutic efficacy of $1\alpha(OH)D_5$ immunoconjugated with c-erbB2 antibody.

3) Study effects of dietary supplementation of $1\alpha(OH)D_5$ on growth and metastasis of human breast carcinomas in experimental animals.

Experimental Design, Methods, Results

<u>Specific Aim 1</u>: Determine whether $1\alpha(OH)D_5$ will alter morphological, biological, and functional characteristics of malignant breast cells and differentiate them to normal condition.

During the process of cell differentiation, malignant cells alter their phenotypic characteristics such as expression of various cell surface proteins. E cadherin, along with catenins (α, β) and integrins ($\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$), are the major cell surface proteins

involved in the interaction of breast cell with its microenvironment.[21-27] The changes in expression of these surface proteins alter the biological and functional characteristics of the cell (such as ability to form cell aggregates or cell-to-cell attachment) and to interact with extracellular matrix proteins.[23-24] Differentiated malignant cells may lose the ability to invade through the extracellular matrix and stromal compartments, and to develop tumor when transplanted into experimental animals. In the present study, we evaluated the effects of $1\alpha(OH)D_5$ on various breast carcinoma cell lines, namely, MDA-MB-231, UISO-BCA-4, MCF-7, and BT-474 cells. MDA-MB-231 and BCA-4 cells are estrogen receptor-negative; MCF-7 and BT-474 cells are receptor-positive. Cells were incubated at 37°C for 10 days in basal culture medium, or in medium containing $1\alpha(OH)D_5$ at 0.1-1 μM concentration. Basal medium used is Minimal Essential Medium containing 5% charcoal-stripped serum. Based on our previous results, all cells treated with vitamin D analog were anticipated to be differentiated at the end of incubation.

Effect of $1\alpha(OH)D_5$ on in vitro growth of breast cancer cells: We first determined response to $1\alpha(OH)D_5$ in various established human breast carcinoma cell lines. Cells (approximately 10,000-15,000/well) were plated in 24-well tissue culture plates. After 24 hours, media were changed. Control cells received MEM-E containing 5% charcoal-stripped serum alone; experimental cells received 10^{-6}M $1\alpha(OH)D_5$ in MEM-E containing 5% charcoal-stripped serum. Medium was changed on days 4 and 7 of initiating treatment. On day 10, the number of cells was counted using a coulter counter. The number of cells in the control group was considered 100%. Data represent mean + SE of control value. Each group contained quadruplet observations.

As shown in Figure 1, $1\alpha(OH)D_5$ significantly inhibited growth of MCF-7, ZR-75-1, UISO-BCA-4, and T47D cells. No growth inhibitory effect was observed in other cell lines studied.

We further examined whether the effect of $1\alpha(OH)D_5$ is transient. UISO-BCA-4 cells were first treated with/without $1\alpha(OH)D_5$ for 10 days as mentioned above. After 10 days, all cells were fed with regular MEM-E medium containing 15% FBS and were further allowed to grow for 10 days. At the end of 10 days, the number of cells was counted in each treatment and control group. Data represent % of control growth. Lane 1 represents the initial number of cells plated (100%); lane 2 represents the number of cells following 10 days of $1\alpha(OH)D_5$/D3 treatment; lane 3 represents % of cells allowed to recover for 10 days following 10 days $1\alpha(OH)D_5$ or D3 treatment. Results are shown in Fig. 2.

Effect of $1\alpha(OH)D_5$ on cell morphology: We examined the effect of $1\alpha(OH)D_5$ on the morphological characteristics of breast cancer cell lines. Following 7-10 days exposure to $1\alpha(OH)D_5$, typical morphological changes were observed in all breast cancer cell lines studied. For example, as shown for UISO-BCA-4[28] cells treated with $1\alpha(OH)D_5$ or 1,25 (OH)2D3, cytoplasm appeared thin and lacy and contained numerous vacuoles. Occasionally, numerous fragmented nuclei were observed. MCF-7 and BT-474 cells showed similar morphological features following vitamin treatment.

Effect of 1α(OH)D_5 on differentiation markers: ICAM-1, nm23, e-cadherin, catenins (α, ß), casein, cytokeratins (CK5, CK-8,CK14, CK-18), vimentin, and integrins ($\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$) are known markers for breast cell differentiation.[22-29] In the present study, we aim to assay all these markers in vitamin D analog-treated cells and control cells by immunohistochemical method, flow cytometric analysis, and Western blot analysis.

We studied the expression of various differentiation markers in four different human breast carcinoma cell lines: UISO-BCA-4, MCF-7, MDA-MB-231, and ZR-75-1. For immunohistochemical studies, cells were plated on coverslips (Nunc Corp., Naperville, Illinois), then incubated for 7 days in the culture medium containing 1α(OH)D_5 (10^{-7}M, 10^{-6}M) or 1α(OH)2 D_3 for 7 days. These compounds were added to medium containing charcoal-stripped serum. At the end of incubation, coverslips were rinsed in PBS, and either processed for immunohistochemical staining or fixed in 10% buffered formalin, 70% ice cold methanol, and acetone, then processed for immunohistochemical staining.

For FACS analysis, cells growing in the culture flasks were incubated with 1α(OH)D_5 or 1α(OH)2D_3 for 7 days. At the end of incubation, cells were harvested, rinsed with PBS, and incubated with appropriate primary antibody or IgG as control for one hour. Cells were rinsed with PBS and incubated with fluorescent-labeled secondary antibody. After thorough washing, cells were fixed in 0.5% buffered formalin and then subjected to FACS analysis. Table 2 summarizes the original (without treatment) status of various biomarkers in cell lines examined by FACS analysis.

Biomarker	UISO-BCA-4	MCF-7	MDA-MB-231	ZR-75-1
Alpha 2 integrin	+	+	+	+
Alpha3 integrin	+	+	Neg/low	+
Alpha5 integrin	+	+	+	Low/neg
Beta 1 integrin	+	+	+	+
Beta 4 integrin	-	+	Low	-
Alpha6 integrin	+	+	+	+
Cytokeratin 8	+	-	-	+/low
Cytokeratin 18	-	-	+	-
Cytokeratin 19	+	+	+	+
ICAM-1	+	+	+	Low/neg
EGFR	-	-	-	-
Insulin-like Growth Factor I	+	+	+	Nd

These results were further confirmed by immunohistochemical studies.

The effect of 1α(OH)D_5 and 1α(OH)2D3 on UISO-BCA-4 cells

We evaluated the effect of 1α(OH)2D_3 (used as a standard vitamin D metabolite) in UISO-BCA-4, ZR-75-1, MCF-7 and MDA-MB-231 cells. As mentioned earlier,

results were evaluated by both immunocytochemistry and FACS analysis. Following treatment with 1α(OH)2D3, we observed enhanced expression of alpha2 integrin, beta 1 integrin, Ck-8, and ICAM-1 proteins. Expression of alpha3 integrin was reduced following 1α(OH)2D3 treatment. Results in UISO-BCA-4 cells are shown in Fig. 3.

Effect of 1α(OH)D_5 was similar to that of 1α(OH)2D3. We observed enhanced expression of alpha2, beta 1, and ck-8 proteins (Figure 3). Changes in the expression of alpha2 integrin following 1α(OH)D_5 treatment were dose-dependent (Figure 4). These results were further confirmed by immunohistochemical staining for alpha2 integrin (Figure 5).

Effect of 1α(OH)D_5 in MCF-7 cells:

We evaluated the effects of 1α(OH)D_5 and 1α(OH)2D3 on MCF-7 cells. Our flow cytometry and immunohistochemical data suggest that both these vitamin D analogs induced ICAM-1, CK-8, alpha2, and beta 1 integrins in these cells.

The effect of 1α(OH)D_5 in MDA-MB-231 cells

We examined the effects of 1α(OH)D_5 and 1α(OH)2D3 in VDR-ER- MDA-MB-231 cells on alpha2, alpha5, beta1, ck-8, ck-18, ck-19, and ICAM-1, alpha6 expression.. We failed to observe significant changes in the expression of any of these biomarkers following 1α(OH)D_5 treatment (10-7M). Fig. 6 shows expression of various biomarkers in control and 1α(OH)D_5-treated MDA-MB-231 cells (Figure 6).

Effect of 1α(OH)D_5 on intacellular lipid accumulation

We determined the effect of 1α(OH)D_5 on the accumulation of lipids as an indicator of differentiation of breast cancer cells. Cells were incubated in the control medium or medium containing 1α(OH)D_5 stained for lipid by oil red o reagent. As shown in our published report, 1α(OH)D_5 enhanced the accumulation of lipid in UISO-BCA-4, T-47D, MCF-7 and ZR-75-1 cells. No such effect was observed in other cell lines studied.

<u>Specific Aim 2</u>: Evaluate the therapeutic efficacy of 1α(OH)D_5-conjugated with c-erbB2. Whether conjugated 1α(OH)D_5 is more effective than c-erbB2 antibody or 1α(OH)D_5 treatment alone.

Preparation of 1α(OH)D_5-c-erbB2 antibody conjugate: For linking the Her-2 antibody to 1α(OH)D_5 we used sulfosuccinimidyl 6 -[4'-azido-2'nitrophenylamido] hexanode (Sulfo-SANPAH) as a linker. It has a molecular weight of 492.4, and the spacer arm linking the antibody and 1α(OH)D_5 is approximately 18.2 A. Antibody against extracellular domain of Her-2/neu (Clone G6.10, Ab-2) was obtained as BSA-free, azide-free solution from Neomarkers (Union City, CA). This antibody is directed against the protein backbone of the extracellular domain of the neu protein. Sulfo-SANPAH cross linker was obtained from Pierce Biotech Company, Rockford, IL. Conjugation of 1α(OH)D_5 will be performed by a two-stage method based on photoaffinity cross linking as described by the supplier of the cross linker. The cross

linker was initially dissolved in DMSO and then was diluted to different molar concentrations (1-10) in phosphate buffer (PH 7, 0.150M). For initial characterization, we used iodinated neu antibody at different concentration. Antibody + cross linker solution was first incubated on ice for 60 minutes in the dark. Following incubation, reaction mixture was dialyzed and the second step reaction was continued. This reaction brings the photoreactive group of the linker face outward from the surface of the modified molecule. In a second step, this photoactivated molecule was coupled by photoactivation to $1\alpha(OH)D_5$. Photoactivation was performed by exposing the reaction mixture with 6 bright camera flashes held approximately 2-3 inches above the reaction vessel. The nitro substituted arylazide group when photolyzed formed an aryl nitrene that can react non selectively to form a covalent bond. Again, the reaction mixture was dialyzed to remove free vitamin D5. Figure 7 shows the structure of $1\alpha(OH)D_5$ linked to Her-2 antibody using Sulfo-SANPAH cross linker.

Identification of $1\alpha(OH)D_5$-Her-2 immunoconjugate using SDS page electrophoresis: For this purpose, ab-2 Her-2 antibody was found to be most suitable for preparation of immunoconjugate. We used different molar concentrations of linker to determine which concentration shows optimal cross-linking with Her-2 neu and $1\alpha(OH)D_5$. Molar ratio used for linker to IgG was 10:1, 30:1, and 60:1. For initial reaction, $1\alpha(OH)D_5$ was used at different concentrations (5-25 µl of 10^{-3}M concentration per reaction). Immunoconjugate (2-10 µl) was subjected to SDS polyacrylamide gel electrophoresis using 4-20% gradient gel. Gel was overlaid over photographic X-ray films and exposed for 24 hours. Antibody was linked to $1\alpha(OH)D_5$. Iodinated antibody showed mobility around 150 kd molecular weight, whereas the immunoconjugate migrated at a shorter distance, at the position of higher molecular weight. The immunoconjugate appeared to have varying molecular weight (it showed smeared pattern between 150 Kd and 250 or higher molecular weight). In conclusion, the linker to antibody ratio (60:1) used in the reaction showed synthesis of immunoconjugate with $1\alpha(OH)D_5$.

$1\alpha(OH)D_5$ was stable after exposure to 3-9 camera flash lights: Generally, compounds structurally related to vitamin D are photosensitive and degrade to a certain extent following exposure to light sources. In order to determine the stability of $1\alpha(OH)D_5$ during camera flash light activation we made $1\alpha(OH)D_5$ solution in phosphate buffer and then exposed it to camera flash lights for 3-9 times. The reaction mixture was extracted with methanol and then subjected to HPLC analysis. $1\alpha(OH)D_5$ processed in the same manner but not exposed to lights was used as an experimental control. As shown in Figure 8, camera flash light used for the preparation of $1\alpha(OH)D_5$ -Her-2 linker did not degrade the $1\alpha(OH)D_5$ (Figure 8).

$1\alpha(OH)D_5$ is functionally active after exposure to light during immunoconjugate biosynthesis: $1\alpha(OH)D_5$ photoexposed for 3-9 times was functionally active. It increased the expression of alpha2 integrin expression in UISO-BCA-4 cells (results similar to that of authentic $1\alpha(OH)D_5$, when cells were exposed for 7 days at 1 µM $1\alpha(OH)D_5$ concentration) (Figure 9).

1α(OH)D_5-Her-2 conjugate shows specific binding to Her-2-expressing human breast cancer cells: We examined the binding of Her-2 alone and 1α(OH)D_5 linked to Her-2 to breast carcinoma cells (BT-474 and ZR-75-1) with different Her-2 status and to other Her-2-negative (HT-1080, UISO-Mel-2) human cancer cell lines. BT-474 cells are known to show overexpression of Her-2/neu, and ZR-75-1 cells have low to moderate expression of Her-2/neu. For the binding study, cells(20,000 cells/well) were seeded in 96-well tissue culture plates, and incubated overnight at 37°C in the atmosphere of 5% CO_2 and 95% air. Following 24-hour incubation, media was aspirated, and 0.2 ml of PBS containing 1 million cpm of ^{125}I Her-2 alone or ^{125}I 1α(OH)D_5 –Her-2 immunoconjugate. Our results are represented in Figure 10. In cells known to express low or undetectable Her-2, we failed to observe Her-2 or immunoconjugate binding. In BT-474 and ZR-75-1 cells, Her-2 and immunoconjugate binding was evident. Binding of immunoconjugated Her-2 was reduced 47% as compared to that of Her-2 alone in BT-474 cells. These results suggest that, by conjugating 1α(OH)D_5, probably some of the binding sites of Her-2 are masked, resulting in reduced binding to BT-474 cells.

1α(OH)D_5–Her-2 conjugate competes with ^{125}I labeled Her-2 for receptor binding sites in BT-474 cells: In order to determine whether ^{125}I 1α(OH)D_5 competes for unlabelled Her-2 antibody for the receptor binding sites, we incubated BT-474 cells with ^{125}I Her-2 antibody in the presence or absence of cold 1α(OH)D_5 –Her-2 conjugate or cold Her-2. As shown in Figure 11, both cold Her-2 antibody and 1α(OH)D_5-Her-2 conjugate were able to compete with ^{125}I Her-2 for receptor binding sites on BT-474 cells.

Biodistribution of ^{125}I Her-2/immunoconjugate to various tissues in vivo in athymic mice: In order to determine specific targeting of breast cancer cells with Her-2 antibody, we used the athymic mice xenograft model. Two different human breast carcinoma cell lines (Her-2-positive BT-474 and Her-2-negative MDA-MB-231) were used for this purpose. Cells (1 million/animal) were injected into 4- to 6-week-old female athymic mice. Tumors at the site of injection were allowed to attain at least 1.0 cm diameter in size. Animals were injected i.p. with ^{125}I Her-2 antibody. Each group consisted of 6 animals. Animals were sacrificed 24 hours after antibody injection, and accumulation of radioactivity was determined in tumor and other visceral organs. In general, tumors with overexpression of Her-2 (BT-474) had significantly higher (300 fold) accumulation of ^{125}I antibody as compared to those tumors with Her-2 negative tumors from MMDA-MB-231 cells (data not shown). We also observed a significant amount of radioactivity in other visceral organs; however, it was not different in animals with Her-2-negative or Her-2-positive tumors. In the second set of experiments, we injected (n=5) animals with Her-2 antibody alone or with 1α(OH)D_5 (n=2) conjugated to ^{125}I Her-2 antibody (approximately 2 million cpm/animal) in animals bearing BT-474 xenografts. Animals were sacrificed 48 hours following injection of the antibody, and radioactivity was counted in preweighed organs using a gamma counter. Figure 12 shows distribution of iodinated antibody/1α(OH)D_5 linked antibody in the tumor and various organs. Radioactivity is normalized as a ratio of radioactivity in the tissue /radioactivity in the muscle (organ showing lowest Her-2 expression). Both Her-2

antibody alone and 1α(OH)D_5 conjugated Her-2 showed maximum accumulation in the tumor. Radioactivity was also detected to a lesser extent in the spleen, kidney, and lung.

Internalization of immunoconjugate in the cells: In order to determine internalization of Her-2 antibody, we have used BT-474 cells. In brief, BT-474 cells were plated on sterile glass coverslip and allowed to attach for 72 hours. Following attachment, cells were incubated in medium containing Her-2 antibody alone or 1α(OH)D_5–Her-2 antibody conjugate (1.5 μg/ml antibody concentration). After the indicated time, cells were fixed in 10% buffered formalin and methanol then incubated with FITC-linked antimouse secondary antibody for 20 min. Coverslips were mounted, and fluorescence was detected using a fluorescence microscope.

Figure 13 shows immunofluorescence following incubation of cells with Her-2 antibody for 0-24 hours. We observed distinct Her-2 antibody localization in BT-474 cells at 20, 30, and 60 min. post incubation with Her-2 antibody. At 2 hr, cell surface localization of Her-2 antibody was less evident, but intracellular localization was observed. By 24 hours, antibody immunoreactivity was observed only in the cytoplasm mostly localized in the vesicular form.

We also examined whether Her-2 antibody incubation of BT-474 cells influences phosphorylation of the Her-2 protein. Cells incubated for 0-24 hours with Her-2 antibody were examined for the phosphorylated form of Her-2 receptor using specific antibody against phospho Her-2. As shown in Figure 13, up to 10 min. phosphorylated antibody was found localized on the membrane; by 2h, most of the immunoreactivity was found to be localized in the cytoplasm, and by 24 hours perinuclear localization of the phospho Her-2 was observed. Thus Figures 13 and 14 collectively suggest that incubation of cells with Her-2 antibody internalize the phosphorylated Her-2 that is bound to Her-2 antibody.

The next question we addressed is whether 1,25(OH)2D5, 1α(OH)D_5, Her-2 antibody, or a combination of Her-2 antibody + D5 treatments influences the expression of Her-2 on BT-474 cells. BT-474 cells were incubated for 0-24 hours with control medium (MEM-E containing 5% SS medium), medium containing 1,25 (OH)2D_3 (10^{-7}M), 1α(OH)D_5 (10^{-6}M), or Her-2 antibody (1.5 μg/ml), or a combination of Her-2 antibody + 1α(OH)D_5. Expression of total Her-2 protein on the cells was determined by incubating the cells with Her-2 antibody, and then with FITC-labeled antimouse antibody. Results are shown in Figure 15. We observed enhanced expression of Her-2 receptors, mostly localized on the cell surface in cells incubated in the control medium for 24,hours. In the cells incubated with 1,25 (OH)2D_3 and 1α(OH)D_5, Her-2 expression was found to be lower than that in the control cells. On the other hand, in cells incubated with Her-2 antibody and Her-2 + D5, immunoreactivity was observed mostly localized to the cytoplasm as observed earlier in Figures 13 and 14. Similar results were obtained by western blot analysis in treated BT-474 cells (Figure 14b). These results show that 1α(OH)D_5 treatment does not influence the transportation of the Her-2 antibody bound to Her-2 receptor in the cytoplasm.

In order to determine whether the 1α(OH)D_5-Her-2 antibody immunoconjugate binds to cells, gets internalized, and behaves similarly as native Her-2 antibody, cells were incubated with immunoconjugate for 0-24 hours. At the end of incubation, binding of immunoconjugate was determined by incubating the cells with FITC-labeled secondary antibody. Results are presented in Figure 16. Our results show that, at 60 min after incubation with immunoconjugate, Her-2 antibody is localized at the cell surface. By 2 hr, some fluorescence was detected intracellularly in the cytoplasm, and 24 hours later intense Her-2 immunoreactivity was observed in the cells intracellularly. Some vesicular Her-2 activity was also evident at this time. As compared to those cells incubated with Her-2 antibody alone, immunoreactivity in immunoconjugate-treated cells was slightly weaker. It is quite possible that secondary antibody binding to Her-2 antibody is reduced by immunoconjugating D5; thus weaker signal is observed in immunoconjugated cells than Her-2 antibody-treated cells. However, our results suggest that the immunoconjugate is able to bind to Her-2 receptor and it is internalized.

Does immunoconjugate bind to VDR? Is immunoconjugate effective in inducing alpha2 integrin expression?

Currently, we are investigating whether VDR binds to the immunoconjugate in breast cancer cells. Even though we have not yet addressed this question, results from our study suggest that the immunoconjugate binds to VDR. BT-474 cells were incubated for 7 days in control medium, medium containing 1α(OH)D_5, Her-2 antibody, Her-2 + 1α(OH)D_5, or the 1α(OH)D_5-her-2 antibody immunoconjugate. At the end of incubation, cell lysates were subjected to western blot analysis; VDR antibody was used as a primary antibody. Our results are shown in Fig. 16b. We observed a specific 49kda protein band in all treatments except the immunoconjugate treatment. In cells treated with IMC, in addition to 49kda, a very high molecular weight protein immunoreactive to VDR was observed. These results indicate that the immunoconjugate used in our assay probably binds to VDR, which is reactive to VDR antibody in this analysis.

Currently, we are evaluating the possibility of VDR interaction with the immunoconjugate by the immunoprecipitation method using VDR antibody. We will immunoprecipitate VDR from control and immunoconjugate-treated cells using VDR antibody, and then analyze by western blot analysis whether Her-2 1α(OH)D_5 is immunoprecipitated along with VDR by VDR antibody. We are also evaluating changes in α2 integrin expression following treatment with the immunoconjugate.

Effect of 1α(OH)D_5 -Her-2 immunoconjugate on in vivo growth of BT-474 cells

We determined the effect of 1α(OH)D_5-Her-2 immunoconjugate on in vivo growth of BT-474 cells transplanted into athymic mice. Immunoconjugate was administered i.p. once a week at antibody concentration equivalent to 5 μg/animal. We also included in the same experiment groups receiving 1α(OH)D_5 in the diet, or Her-2 antibody treatment + D5-supplemented diet. Each group consisted of 5 animals; control group consisted of 3 animals. Mean tumor volume in each treatment group on days 21 and 56 after cell inoculation is shown in Figures 17 and 18. Our results clearly show that

the immunoconjugate is more effective in inhibiting the tumor growth than Her-2 antibody alone.

Specific aim 3: Study effects of dietary supplementation of 1α(OH)D_5 on growth and metastasis of human breast carcinomas in experimental animals.

The effects of 1α(OH)D5, Her-2, and 1α(OH)D5 + Her-2 antibody were determined on BT-474 and ZR-75-1 cells transplanted into athymic mice. The cells (1 million/animal) were suspended in a mixture of HBSS: Matrigel (1:1 vol/vol) then injected s.c. into the dorsal flank region of 3- to 4-week-old female Balb/c athymic mice. All animals received s.c. estrogen pellet (0.72 mg/animal, 60 days release, Innovative Research, Saratoga, FL). Animals were divided into various groups: 1) receiving regular powdered diet mixed with ethanol as vehicle; 2) receiving 1α(OH)D_5 (12.5 μg/kg diet)-supplemented powdered diet; 3) receiving i.p. injection of Her-2 antibody (5 μg/animal, once weekly) and regular powdered mouse diet as in group 1; 4) receiving Her-2 antibody (5 μg in 0.1 ml saline/animal, once weekly) i.p. as in group 3 but receiving diet supplemented with 1α(OH)D5. Groups 1 and 2 also received i.p. injection of saline (0.1 ml, once weekly).

Both control and 1α(OH)D5-supplemented diet were given to the animals in sterile food cups; an equal amount of food was placed in each cup. Food cups were protected from direct light exposure. Food cups were changed twice weekly. At the time of food cup change, diet consumption in each group was estimated roughly based on unused food contents in the cups. All animals received water ad libitum. Each group consisted of a minimum of 5 animals. Animals were weighed and examined once weekly for growth of tumor at the site of injection. Once a palpable tumor developed, tumor size was monitored using calipers. Tumor volume was calculated as cm^3 using the formula, tumor volume = 3.14/6 x length x width x depth. Data represent mean + SE tumor volume (cm^3) in each group. Animals were sacrificed at the indicated time unless they appeared to be moribund or tumors showed sign of necrosis. At the termination, tumors were removed, fixed in 10% buffered formalin, and processed for histopathological and immunocytochemical studies.

Preparation of diet: For preparation of 1α(OH)D_5-supplemented diet, a known amount of 1α(OH)D_5 was dissolved in absolute ethanol and then mixed with powdered mouse chow (Teklad, Madison, WI) using diet mixer. Diet was stored in foiled containers to protect from light and stored at 4°C. Stability of 1α(OH)D_5 was determined periodically. An aliquot of the diet was extracted with methanol, and the extract was subjected to HPLC analysis. Control diet was mixed with ethanol only (equal to that used for 1α(OH)D_5 diet). Ethanol from the diet mixtures was evaporated by placing it in sterile culture hoods at room temperature for 20 min..

The effect of test compounds on in vivo growth of BT-474and ZR-75-1 cells transplanted into athymic mice: We used the nude mouse model to evaluate the effect of 1α(OH)D_5 on *in vivo* growth of BT-474 cells and ZR-75-1 cells. These two cell lines were selected based on expression of Her-2 and their ability to form tumors in the athymic mouse model. All animals received estradiol pellets to enhance the growth of

tumor. Growth curves are shown for mean tumor volume in each group. Growth of BT-474 cells was slow initially in both control and experimental groups. In the group receiving control diet, BT-474 tumors grew at a very slow rate initially for the first 20 days. After 20 days, the tumor attained exponential growth phase; by day 56, the mean tumor volume was 0.2 cm^3. In animals receiving $1\alpha(OH)D_5$, tumors grew at a slower rate during the first 15 days and achieved maximum tumor volume of approximately 0.03 cm^3 and then started to decline. At day 56 in animals whose diet was supplemented with $1\alpha(OH)D_5$, tumor volume was <0.02 cm^3, 10-fold smaller than that in the control group. Similarly, in animals receiving i.p. Her-2 antibody administration, tumors grew larger between days 20 and 56; at day 56, mean tumor volume in this group was 0.11 cm^3. Combined treatment with Her-2 antibody and dietary supplement of $1\alpha(OH)D_5$ showed further reduction in tumor volume compared to those in either $1\alpha(OH)D_5$ or Her-2 treatment alone (Figure 19).

We further examined the histopathology of tumors obtained at the time of termination. As shown in Figure 20, xenografts originated in animals receiving control diet showed highly cellular morphology. In contrast, xenografts originated in animals treated with $1\alpha(OH)D_5$ showed few cell patches embedded in the Matrigel stroma. Xenografts originated in animals treated with i.p. administration of Her-2 antibody were histopathologically similar to those observed in animals receiving control diet without Her-2 antibody treatment. Interestingly, xenograft originated in animals receiving both $1\alpha(OH)D_5$-supplemented diet and Her-2 antibody administration showed few cells embedded in the matrix. Cells showed the morphology of differentiated cells.

We evaluated the effects of $1\alpha(OH)D_5$ and Her-2 antibody on ZR-75-1 cells transplanted in athymic mice. As shown in Figure 21, in the control group receiving regular powdered diet, tumors showed exponential growth from day 10-42. In animals receiving c-neu antibody but normal diet, tumor growth was slower than in the control group receiving no Her-2 antibody and regular diet. The group receiving diet supplemented with $1\alpha(OH)D_5$ had significantly ($p<0.05$) smaller tumor volume than that receiving control diet without $1\alpha(OH)D_5$ supplement. Similarly, mean tumor volume was significantly ($p<0.05$) smaller in the group of animals receiving combined $1\alpha(OH)D_5$ and Her-2 antibody treatment as compared to that observed in the control group, the group receiving Her-2 antibody alone, or the group receiving $1\alpha(OH)D_5$ dietary supplement alone (Figure 20). Histopathological details of the tumors obtained from the animals on $1\alpha(OH)D_5$ diet and those receiving the control diet are shown in Figure 22. Tumor from the control group consisted of tightly packed large epithelial cells with minimum inter cellular stromal component. Tumor cells had large prominent nucleus. In contrast, tumors from the $1\alpha(OH)D_5$-treated group showed the presence of small clusters of cells embedded in the stroma (mostly Matrigel). Cells were smaller in size, and nuclei were smaller than those in the control group. The occasional presence of cell clusters with morphology similar to that observed in control tumor was also observed (Figure 21).

In order to determine whether the growth inhibitory effect of $1\alpha(OH)D_5$ is transient, we switched all experimental groups (animals previously receiving Her-2 antibody alone, $1\alpha(OH)D_5$-supplemented diet, or Her-2 antibody + $1\alpha(OH)D_5$-

supplemented diet) to control regular powdered diet on day 45. None of the groups received Her-2 antibody after day 45. Tumor volume was monitored periodically and recorded. As shown in Figure 23, after switching the animals to regular diet, all groups except the group receiving $1\alpha(OH)D_5$ + Her-2 antibody combination treatment showed accelerated tumor growth. At day 64, when the experiment was terminated, mean tumor volume in animals initially receiving combination treatment was significantly ($p<0.05$) smaller than in the three other groups (Figure 23). These results were further supported by histological examination of tumors in different groups. As shown in Figure 24, the histopathology of tumors in control, $1\alpha(OH)D_5$, or Her-2 antibody were similar, showing highly cellular histopathology. Tumors in animals who received combination treatment showed only a few cells embedded in stromal tissues.

Serum calcium levels in animals receiving different treatments: We determined the serum calcium levels in animals receiving the different treatment regimens mentioned above. Determination of serum calcium levels: Quantitative determination of serum calcium levels was performed by calorimetric analysis using calcium (Arsenazo III) reagent (Sigma Diagnostics, St. Louis, MO). Calcium in the serum reacts to the Arsenazo III reagent and forms purple-colored calcium-Arsenazo III complex. The intensity of the color is read at 600 nm and is evaluated in relation to the color intensity obtained by processing simultaneously known calcium standards (Sigma Diagnostics, St. Louis, MO).

In animals bearing BT-474 tumors, mean serum calcium levels at the time of experiment termination were not significantly different in control vs. the group receiving $1\alpha(OH)D_5$ diet or only Her-2 antibody. In animals receiving combination therapy (Her-2 antibody + $1\alpha(OH)D_5$), serum calcium levels were higher ($p<0.05$) than those in the control group. Similar serum calcium profiles were observed in animals bearing ZR-75-1 xenograft (Figure 25). In general, no physically apparent symptoms of hypercalcemia were reported in any treatment group. Animals appear to be active, and no signs of lethargy or dehydration were noticed, although mean body weight gain in groups receiving $1\alpha(OH)D_5$-supplemented diet was significantly ($p<0.05$) less than in those groups receiving control diet with no $1\alpha(OH)D_5$ supplement (Figure 26). One week after treatments ceased, body weights did not significantly differ between groups receiving control and $1\alpha(OH)D_5$-supplemented diet. Serum calcium levels were determined in groups of animals after discontinuation of the treatment, at the time of termination of the experiment. No significant difference in the serum calcium levels was observed in various treatment groups (Figure 27).

Reportable outcomes

- **Publications and abstracts:**

1) **Mehta R.R**. Mehta R.G. Differentiation of human breast carcinoma cell line by a novel vitamin D analog: 1α(OH)D_5 Int. J. Oncology, 16, 65-73, 2000
2) Lazzaro G., Agadir A., Qing W., Poria M., **Mehta R R**. Moriarty R.M., Zhang X, Mehta R.G. Induction of differentiation by 1α(OH)D_5 in T47D human breast cancer cells and its interaction with vitamin D receptor. Eur. J. Cancer, 36, 780-786, 2000
3) Mehta R.R. Graves J.M. Effect of combination treatment with Her-2 antibody and 1α(OH)D_5 in human breast carcinoma, Clin Cancer Res., 2000, submitted.
4) Mehta R.R. and Graves J. Modulation of alpha2 integrin by vitamin D analog, to be submitted.
5) Mehta R.R. and graves J.M. Breast tumor cell targeted novel therapy, to be submitted.

Abstracts presented at various meetings

1. Graves J.M., Mehta R.G., **Mehta R.R.**, Antiproliferative effects of 1αhydroxy vitamin D5 in various human breast carcinoma cell lines. 20th Annual San Antonio breast cancer Symposium, 1997.
2. Marler K., Bratescu L., Graves J.M., Das Gupta T.K. **Mehta R.R.** Implication of p53 in Matrigel enhanced growth and metastasis of human breast carcinoma. 20th Annual San Antonio Breast Cancer Symp. 1997.
3. **Mehta R.R.,** Graves J.M., Modulation of surface proteins by 1α(OH)D_5: Induction of breast carcinoma cell differentiation. Proc. Am. Assoc. Cancer Res. New Orleans, LA, 1998.
4. Lazzaro G., Sharpless S., Qing W., **Mehta R.R.,** Das Gupta T.K., Mehta R.G. Study of nm23 gene expression in an N-Methyl –N-nitrosourea transformed human breast epithelial cell line. Proc. Am. Assoc. Cancer. Res. New Orleans, LA, 1998.
5. Kasangra A.M., Qing W., **Mehta R.R.,** Mehta R.G. Induction of Vitamin and TGF beta receptors during differentiation of human breast cancer cells by 1 alpha hydroxy-vitamin D5. Proc. Am. Assoc. Cancer res. New Orleans, LA, 1998.
6. **Mehta R.R.**, Christov K., Hawthorne M., Graves J.M., Parikh K., Mehta R.G. Evaluation of toxicological parameters for 1 alpha hydroxy vitamin D5, a novel analog of vitamin D. Proc. Am. Assoc. Cancer Res. New Orleans, LA, 1998.
7. Rothschild L.G., Kansagra A.M., Graves J., **Mehta R.R.**, Mehta R.G. Suppression of estrogen inducible growth by 1α-hydroxyvitamin D5 is mediated by vitamin D receptors in breast cancer cells. Proc. Am. Assoc. Cancer Res., vol. 40, pp 650, 1999
8. **Mehta R.R.**, Graves J.M., Mehta R.G. Induction of a2- integrins during differentiation of breast cancer cells by 1α(OH)D_5. Proc. Am. Assoc. Cancer Res., vol. 40, pp 650, 1999
9. **Mehta R.R.,** Graves J.M., Das Gupta T.K. Targeting human breast carcinoma cells with 1α(OH)D_5 linked to Her-2 antibody. 91st Annual AACR meeting, April 2000, submitted.

Grants and Funding applications

1. Therapeutic effect of 1α(OH)D_5, NCI, submitted, 2000
2. CTR application, U.S. Army, funded 1999

Conclusions:

- $1\alpha(OH)D_5$ showed both cell-differentiating and growth-inhibitory actions in vitro in human breast carcinoma cell lines. Amongst the panel of human breast cancer cell lines studied, UISO-BCA-4, ZR-75-1, MCF-7, BT-474 and T-47D cells were responsive to the growth-inhibitory and cell-differentiating actions of $1\alpha(OH)D_5$.
- $1\alpha(OH)D_5$ increases the expression of alpha2 integrin, casein, and intracellular lipid.
- In vivo, $1\alpha(OH)D_5$ supplemented in the diet shows growth-inhibitory effect. Cells exposed to $1\alpha(OH)D_5$ show more differentiated morphology.
- $1\alpha(OH)D_5$ (12.5 μg/ml) or Her-2 antibody (1.5 μg/ml) treatment alone given in vitro shows growth inhibition of BT-474 cells. These cells are known to express high Her-2 protein. The combination of Her-2 antibody + $1\alpha(OH)D_5$ is more effective at inhibiting growth of cells than is either agent given alone.
- In vivo $1\alpha(OH)D_5$ supplemented in the diet is effective at reducing the human breast cancer cells translated into athymic mice. However, combination treatment with both $1\alpha(OH)D_5$ and Her-2 antibody is more effective than either treatment alone. Also, combination treatment is more effective in inhibiting tumor growth even after discontinuation of both treatments.
- $1\alpha(OH)D_5$ supplemented in the diet showed no serious effect; however, a hypercalcemic effect was noticed in animals although serum calcium levels returned to control levels within two weeks after discontinuation of the treatment.
- $1\alpha(OH)D_5$-Her-2 immunoconjugate inhibited in vivo growth of BT-474 cells. Immunoconjugate administration did not have any effect on serum calcium levels.

Personnel involved

1. R.R. Mehta, Ph.D., PI
2. J.M. Graves, Research Assistant

Appendices (attached)

- **Figures 1-27**
- **Publications and Abstracts**

1. **Mehta R.R**. Mehta R.G. Differentiation of human breast carcinoma cell line by a novel vitamin D analog: $1\alpha(OH)D_5$ Int. J. Oncology, 16, 65-73, 2000
2. Lazzaro G., Agadir A., Qing W., Poria M., **Mehta R.R.,** Moriarty R.M., Zhang X, Mehta R.G. Induction of differentiation by $1\alpha(OH)D_5$ in T47D human breast cancer cells and its interaction with vitamin D receptor. Eur. J. Cancer, 36, 780-786, 2000
3. Mehta R.R. Graves J.M. Effect of combination treatment with Her-2 antibody and $1\alpha(OH)D_5$ in human breast carcinoma, Clin Cancer Res., 2000, submitted.

4. Graves J.M., Mehta R.G., **Mehta R.R.**, Antiproliferative effects of 1αhydroxy vitamin D5 in various human breast carcinoma cell lines. 20th Annual San Antonio breast cancer Symposium, 1997.
5. **Mehta R.R.,** Graves J.M. Modulation of surface proteins by $1\alpha(OH)D_5$: Induction of breast carcinoma cell differentiation. Proc. Am. Assoc. Cancer Res. New Orleans, LA, 1998.
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7. Kasangra A.M., Qing W., **Mehta R.R.,** Mehta R.G. Induction of Vitamin and TGF beta receptors during differentiation of human breast cancer cells by 1 alpha hydroxy-vitamin D5. Proc. Am. Assoc. Cancer res. New Orleans, LA, 1998.
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9. Rothschild L.G., Kansagra A.M., Graves J., **Mehta R.R.**, Mehta R.G. Suppression of estrogen inducible growth by 1α-hydroxyvitamin D5 is mediated by vitamin D receptors in breast cancer cells. Proc. Am. Assoc. Cancer Res., vol. 40, pp 650, 1999
10. **Mehta R.R.**, Graves J.M., Mehta R.G. Induction of a2- integrins during differentiation of breast cancer cells by $1\alpha(OH)D_5$. Proc. Am. Assoc. Cancer Res., vol. 40, pp 650, 1999
11. **Mehta R.R.,** Graves J.M., Das Gupta T.K. Targeting human breast carcinoma cells with $1\alpha(OH)D_5$ linked to Her-2 antibody. 91st Annual AACR meeting, April 2000,

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Figure 1. Effect of 1μM 1α(OH)D_5 on growth of various breast cancer cell lines. Cells were plated in 24-well plates and incubated for 10 days in culture medium (MEM-E with 5% charcoal-stripped serum) or medium containing 1μ1α(OH)D_5. Medium was changed on days 4 and 7. Data represent mean ± SE of 4 independent observation. % is calculated in relation to control as 100%.

FIGURE 1

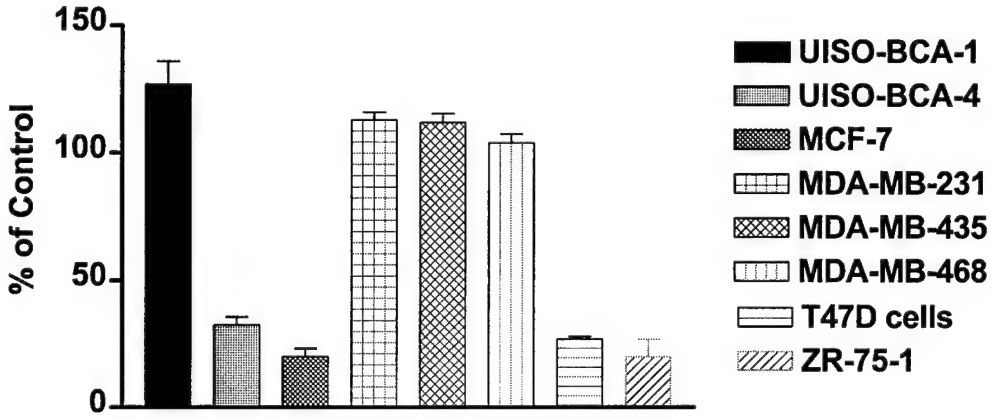
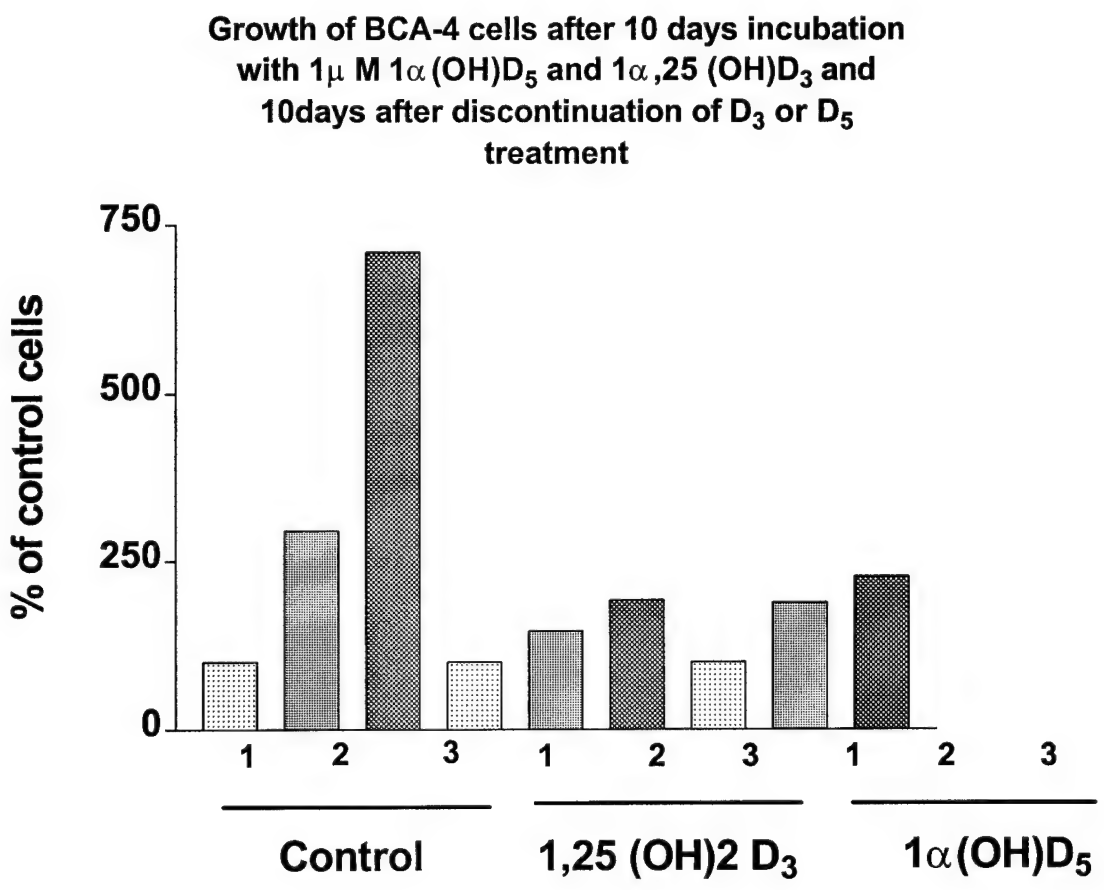

150
100
50
0
% of Control
UISO-BCA-1
UISO-BCA-4
MCF-7
MDA-MB-231
MDA-MB-435
MDA-MB-468
T47D cells
ZR-75-1

Figure 2. Effect of $1\alpha(OH)D_5$ on the growth of UISO-BCA-4 cells: Cells were incubated in stripped serum containing medium (lane 1), $1,25(OH)_2D_3$, or $1\alpha(OH)D_5$ for 10 days (lane 2), or, after 10 days, they were incubated in regular serum containing medium (lane 3). Data represent % of control growth.

FIGURE 2



Effect of 1α (OH)D_5 on growth of UISO BCA-4 cells: lane 1, (100%) cells at initial plating, lane 2, after 10 days incubation in charcoal stripped serum containing medium alone (control) or with 1,25 $(OH)_2$ D3/1α (OH)D_5; lane 3, after treatment as in lane 2, medium was changed to a regular MEM-E containing 15% FBS and cell growth was maintained for additional 10 days. Data represent % (of initial plating) of control cell growth.

Figure 3. Effect of $1\alpha(OH)D_5$ on various cell surface markers in UISO-BCA-4 cells. Analysis was performed using flow cytometry.

FIGURE 3

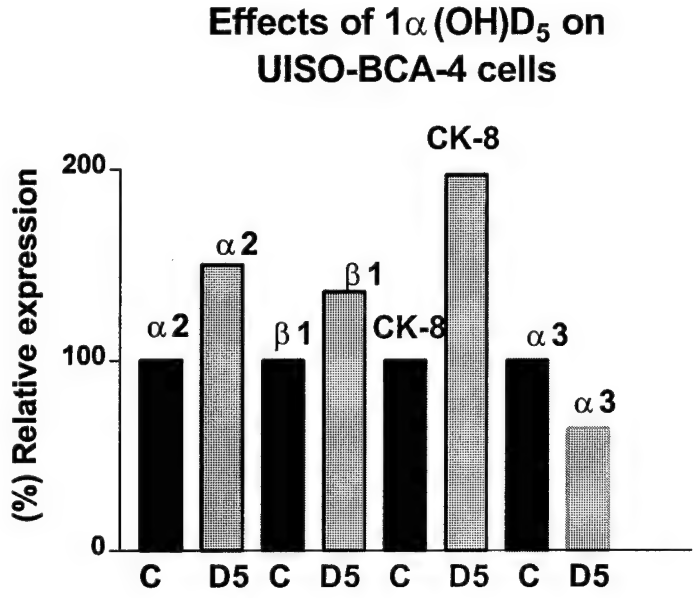
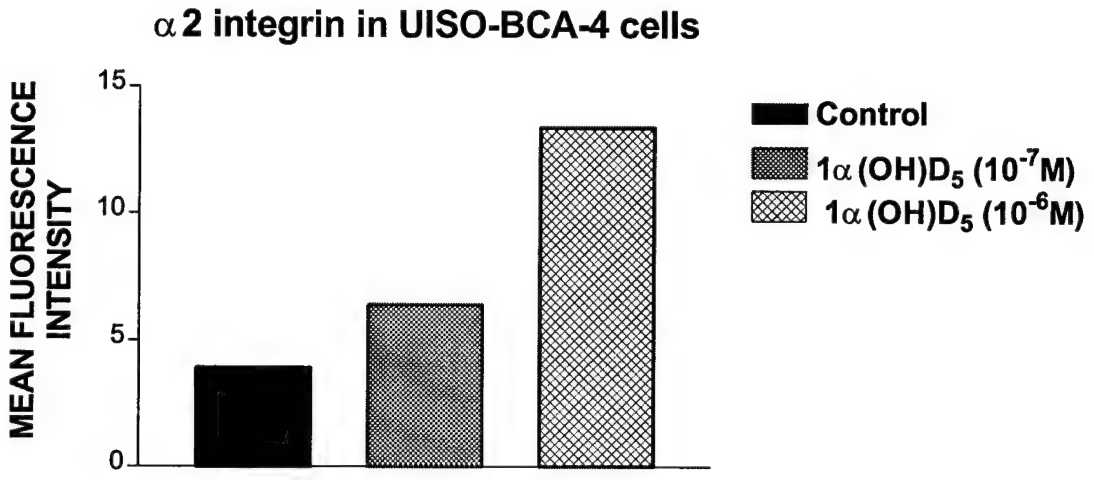

Effects of 1α(OH)D5 on
UISO-BCA-4 cells
(%) Relative expression
200
100
0
α2
α2
β1
β1
CK-8
CK-8
α3
α3
C
D5
C
D5
C
D5
C
D5

Figure 4. Effect of 1α(OH)D5 on α2 integrin expression in UISO-BCA-4 cells. α2 integrin was analyzed by flow cytometry.

FIGURE 4



UISO-BCA-4 cells were incubated in the presence or absence of $1\alpha(OH)D_5$ for 7 days. Expression of α2 integrin was determined by flow cytometry.

Figure 5. Immunohistochemical detection of $\alpha 2$ integrin following in vitro treatment with different concentrations of $1\alpha(OH)D_5$.

FIGURE 5

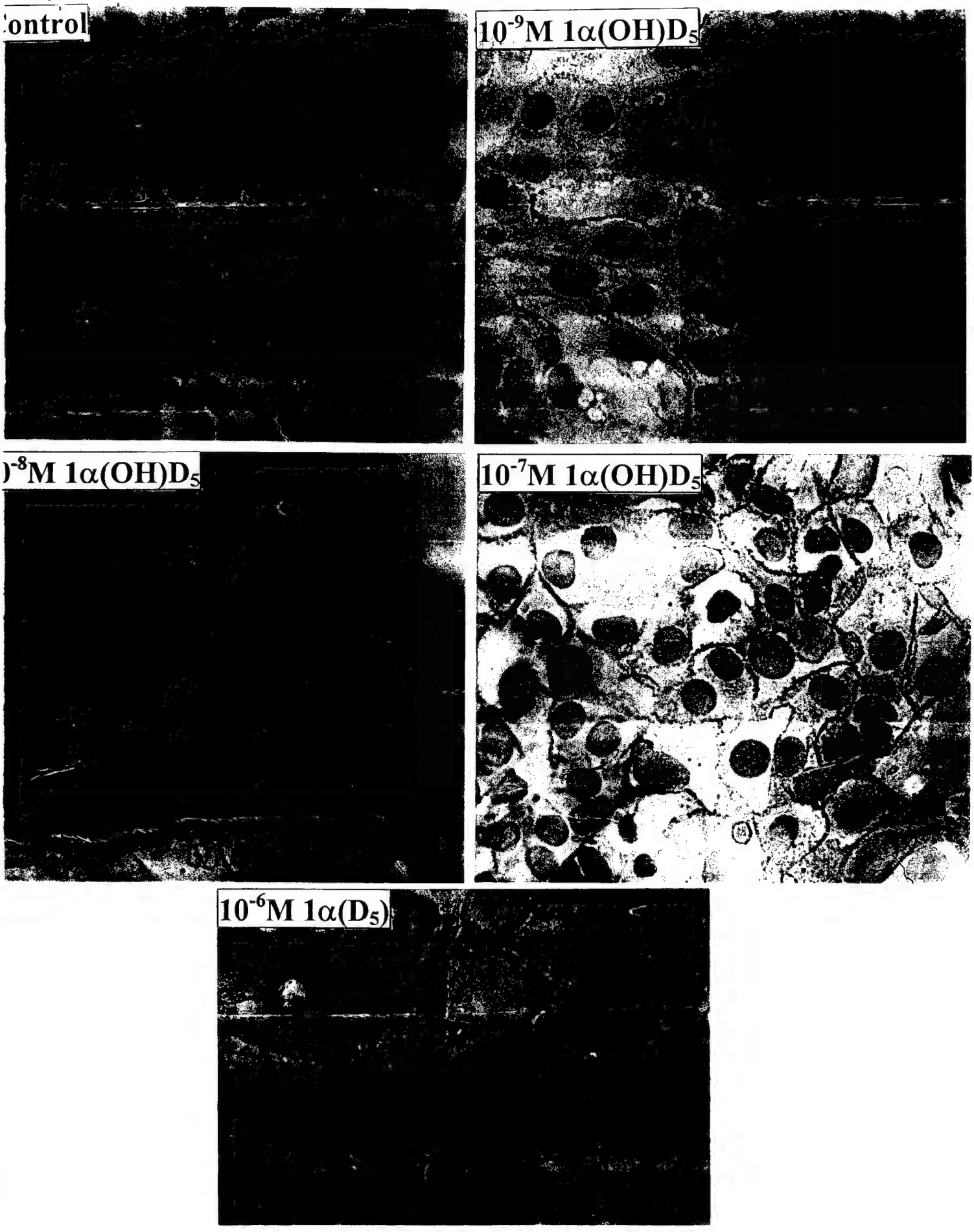


Figure 6. Effect of $1\alpha(OH)D_5$ on expression of various cell surface proteins in MDA-MB-231 cells.

FIGURE 6

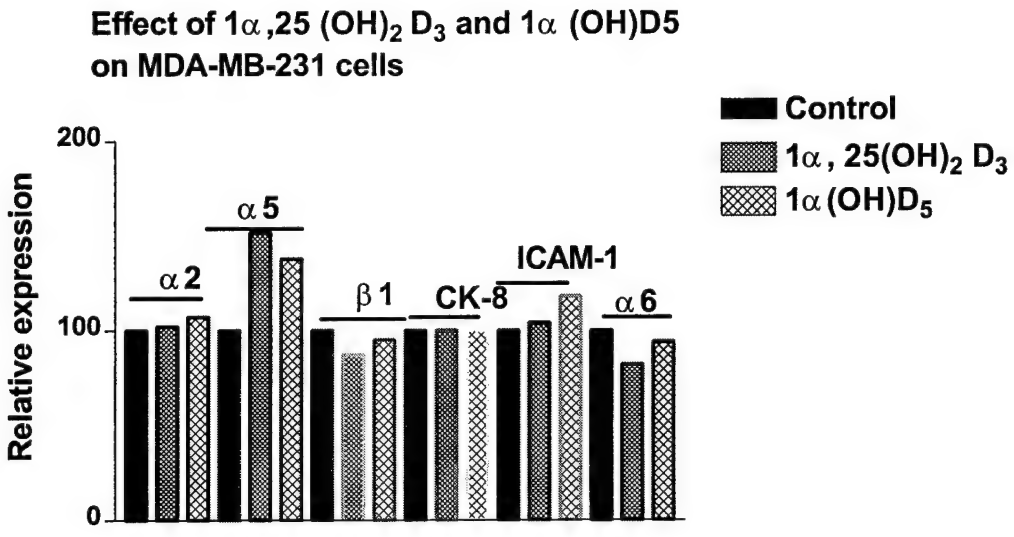
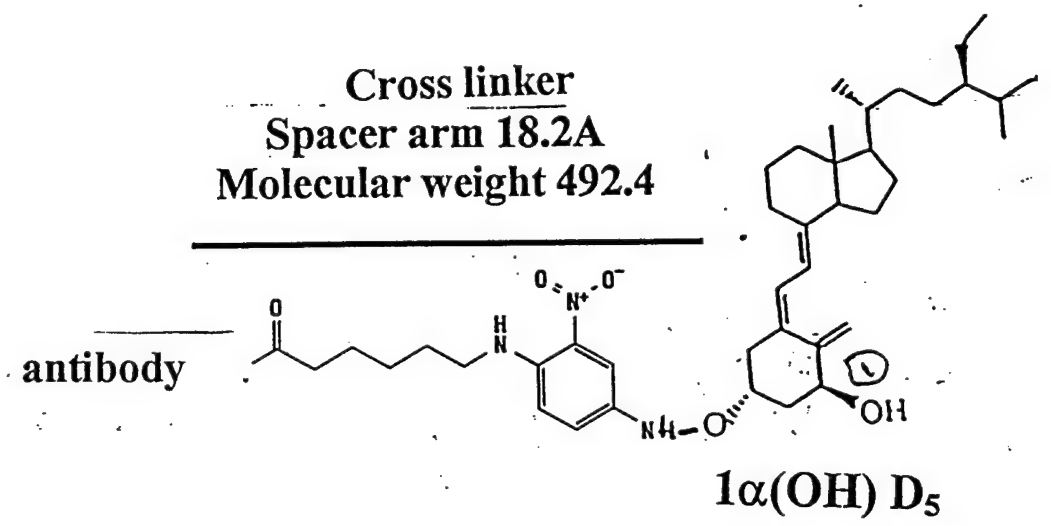

Effect of 1α,25 (OH)2 D3 and 1α (OH)D5
on MDA-MB-231 cells
Control
1α, 25(OH)2 D3
1α(OH)D5
Relative expression
200
100
0
α2
α5
β1
CK-8
ICAM-1
α6

Figure 7. Structure of 1α (OH)D_5-Her-2 antibody linked using SANPAH as a linker.

FIGURE 7

Structure of Her-2 antibody linked to 1α(OH)D_5

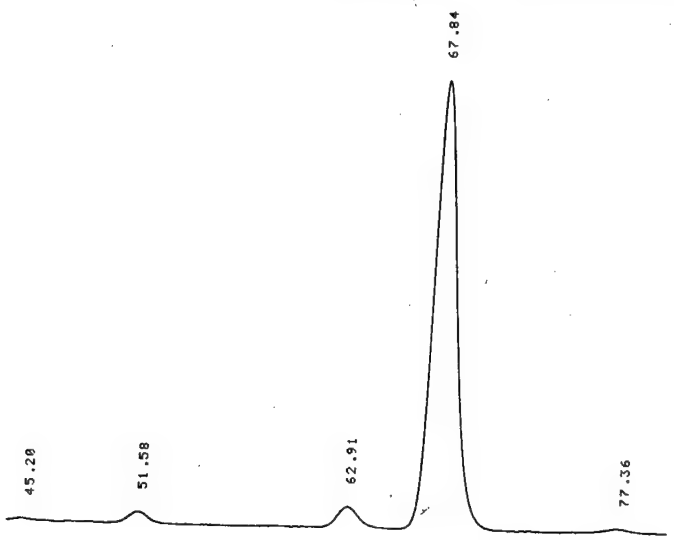


1α(OH) D_5

Figure 8. HPLC analysis of authentic 1α(OH)D5 and the same compound exposed to camera flash lights 6 times.

FIGURE 8

Control - non exposed.



6x exposed by flash light

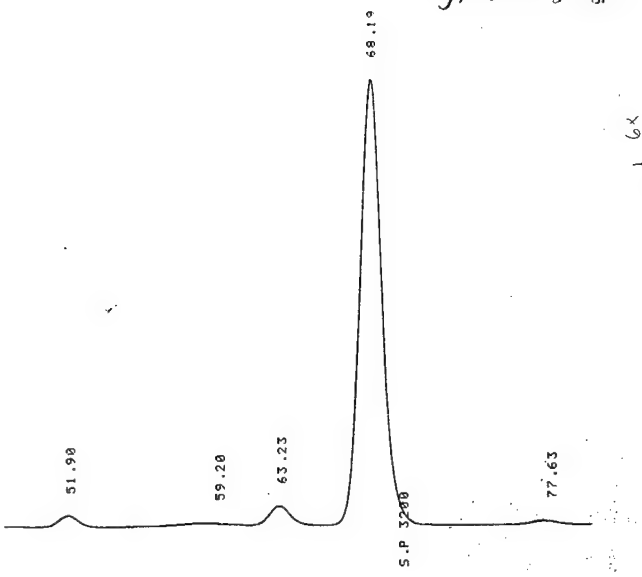


Figure 9. $1\alpha(OH)D_5$ exposed to camera flash light was able to induce $\alpha 2$ integrin in UISO-BCA-4 cells. UISO-BCA-4 cells were incubated with authentic $1\alpha(OH)D_5$ or exposed to camera light for 3-69 times for 7 days. $\alpha 2$ integrin expression was determined by incubating cells with $\alpha 2$ integrin antibody, followed by FITC-labeled secondary antibody. For determination of nonspecific binding, cells were incubated with IgG as a control.

FIGURE 9

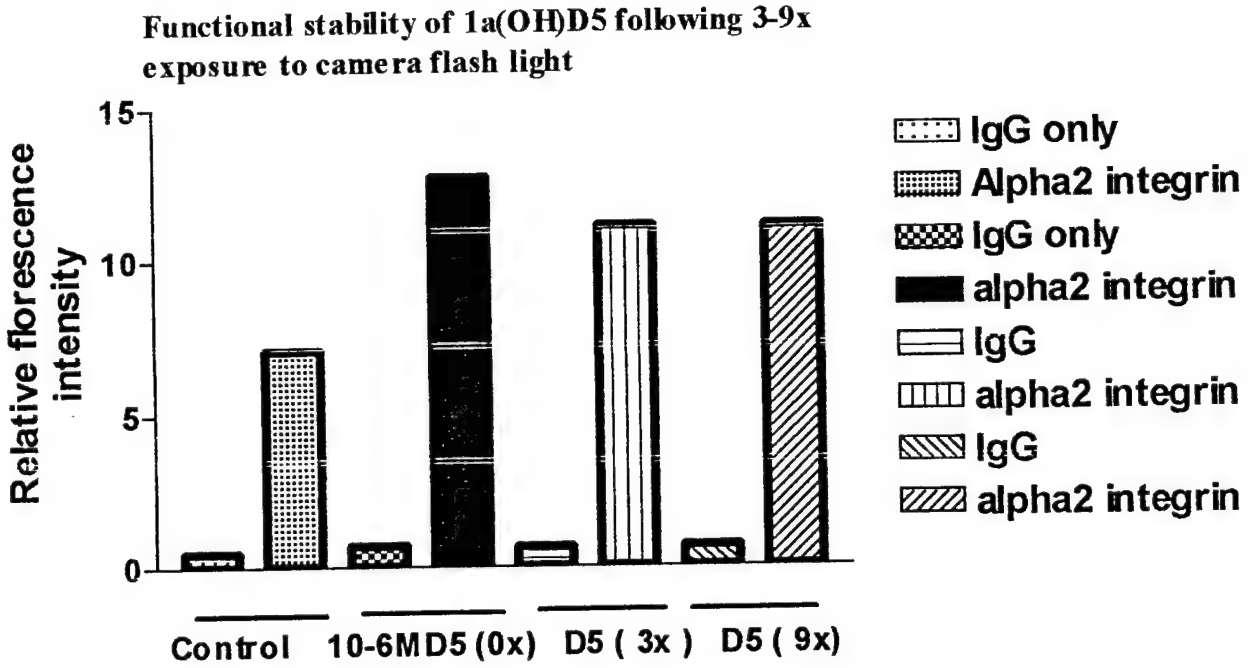
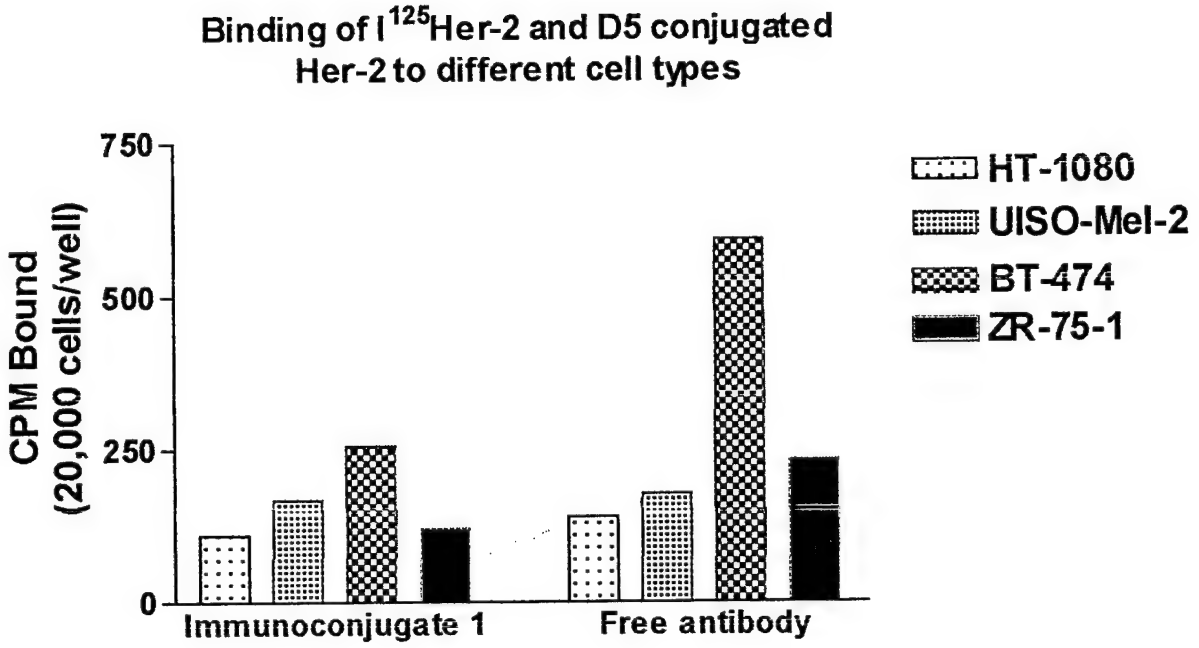

Functional stability of 1a(OH)D5 following 3-9x exposure to camera flash light
Relative florescence intensity
15
10
5
0
Control
10-6MD5 (0x)
D5 (3x)
D5 (9x)
IgG only
Alpha2 integrin
IgG only
alpha2 integrin
IgG
alpha2 integrin
IgG
alpha2 integrin

Figure 10. Binding of ^{125}I Her-2 antibody and 1α(OH)D_5–Her-2 immunoconjugate to different cell types. CPM represent specific binding to the cells. In brief, cells in a monolayer were incubated with ^{125}I-labeled Her-2 or immunoconjugate for 2 hrs at RT. At the end of incubation, cells were washed with buffer, and bound radioactivity was determined using a gamma counter. Nonspecific binding was determined using 100-fold excess unlabeled Her-2 antibody.

FIGURE 10

cells.



binding sites

Figure 11. Competition of immunoconjugate with Her-1 antibody for Her-2 binding sites. BT-474 cells were incubated with ^{125}I- immunoconjugate alone or with increasing concentrations of Her-2 antibody. The amount of radioactivity bound to cells was determined by a gamma counter.

FIGURE 11

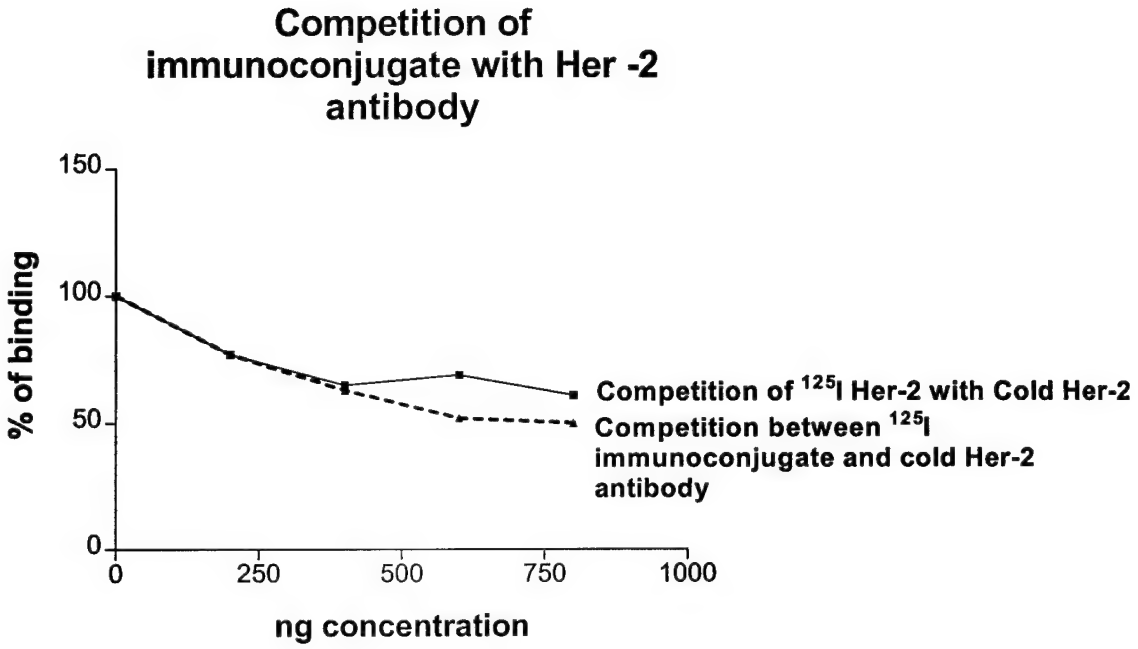

Competition of immunoconjugate with Her -2 antibody
150
100
50
0
% of binding
0
250
500
750
1000
ng concentration
Competition of 125I Her-2 with Cold Her-2
Competition between 125I immunoconjugate and cold Her-2 antibody

Figure 12. Biodistribution of ^{125I} 1α (OH)D_5-Her-2 immunoconjugate in BT-474 cells. Female athymic mice bearing palpable BT-474 xenografts were injected i.p. with ^{125}I immunoconjugate. Animals were sacrificed 48 hours later, visceral organs were excised, and the radioactivity was determined using a gamma counter. Data represent relative radioactivity as tissue-to-blood ratio.

FIGURE 12

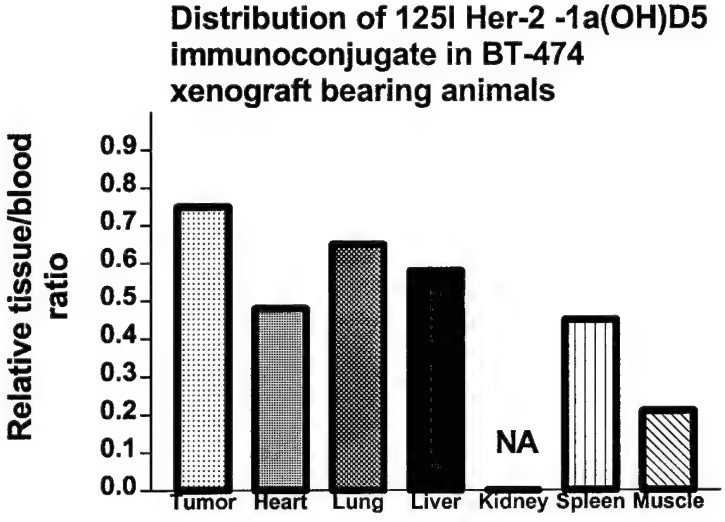
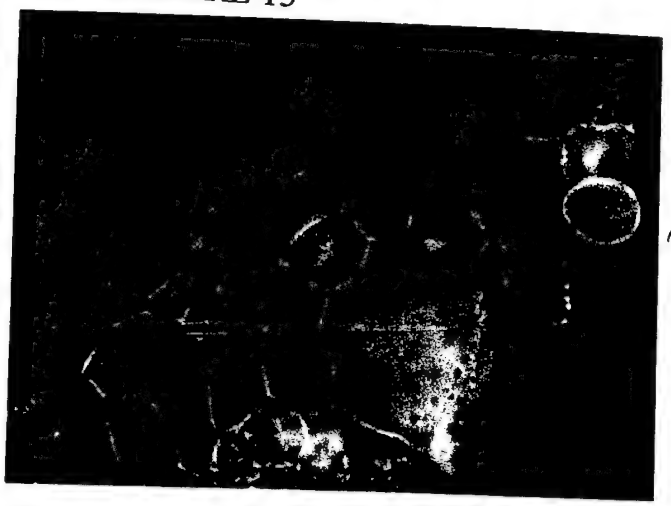

Distribution of 125I Her-2 -1a(OH)D5 immunoconjugate in BT-474 xenograft bearing animals
Relative tissue/blood ratio
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
NA
Tumor
Heart
Lung
Liver
Kidney
Spleen
Muscle

Figure 13. Internalization of Her-2 antibody in BT-474 cells. Cells plated on sterile glass coverslips were incubated with Her-2 antibody (1.5 μg/ml) at 37°C for the indicated time. At the end of incubation, cells were fixed in formalin and then incubated with FITC labeled secondary antibody and mounted.

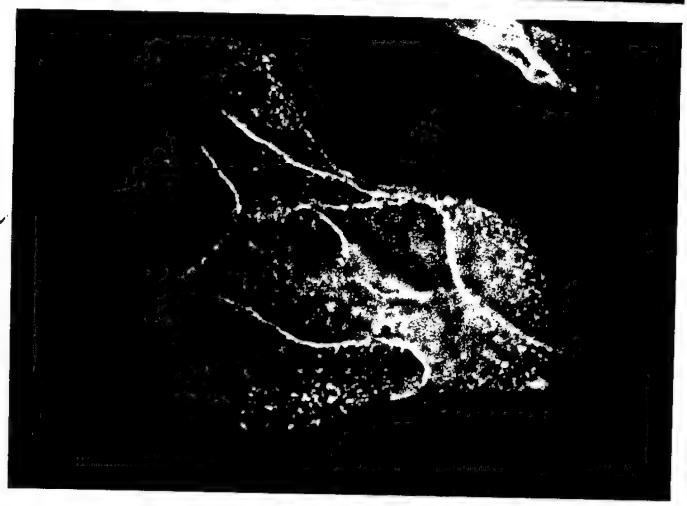
Internalization of Her-2 Ab

FIGURE 13

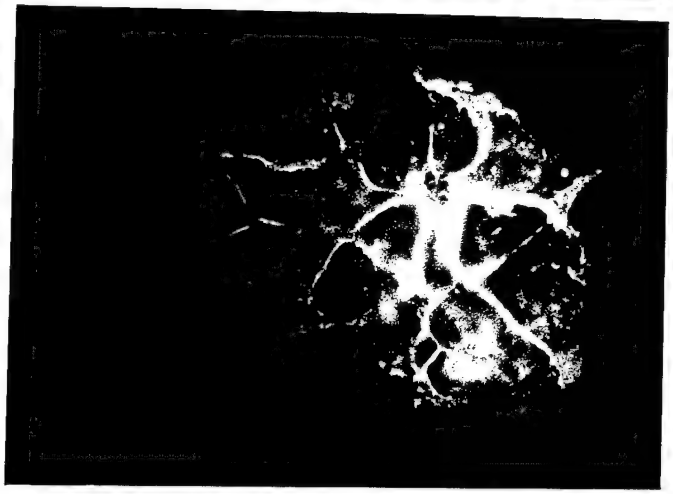
0min



10 mi

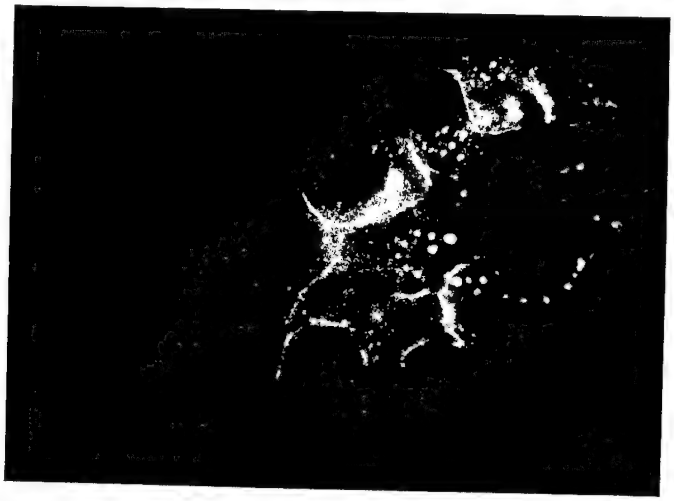


20min

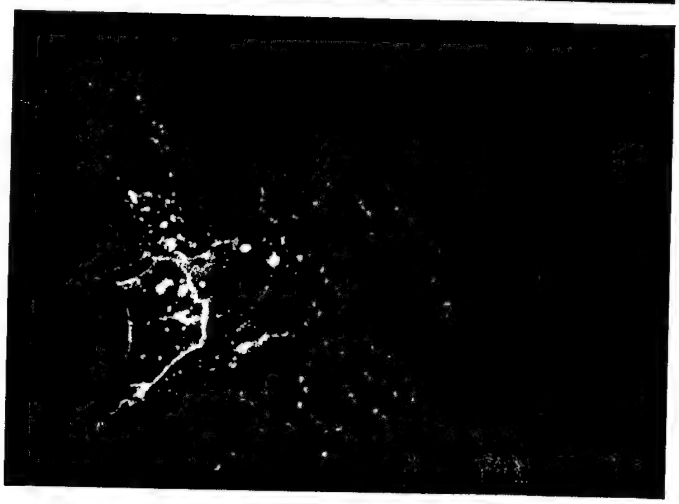


60mi

120 min



2h



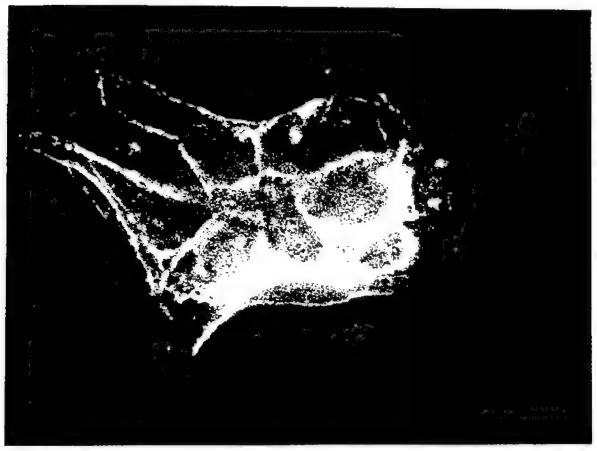
4h

24h

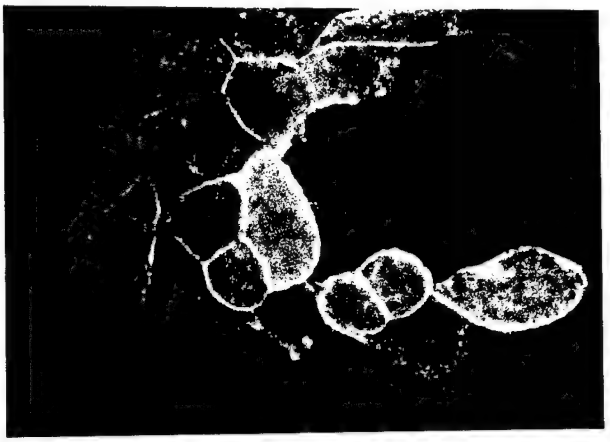
Figure 14. Detection of phosphorylated Her-2 antibody following incubation of BT-474 cells with her-2 antibody. Cells were incubated with Her-2 antibody as mentioned in Figure 13 for the indicated time, at the end of incubation cells were fixed and then incubated with phospho specific Her-2 antibody, secondary FITC labeled antibody. Fluorescence was visualized under microscope.

Phosphorylated Her-2 Ab (Time course)

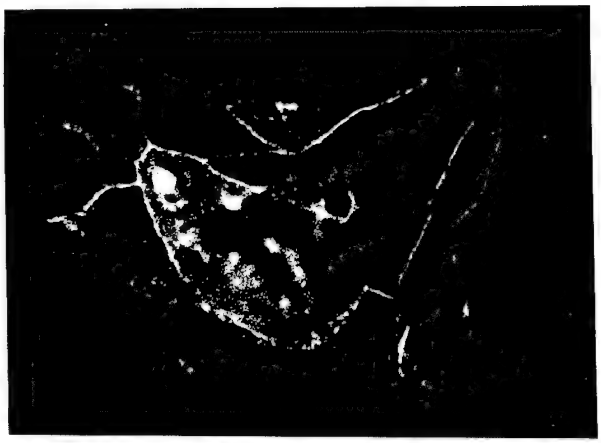
FIGURE 14



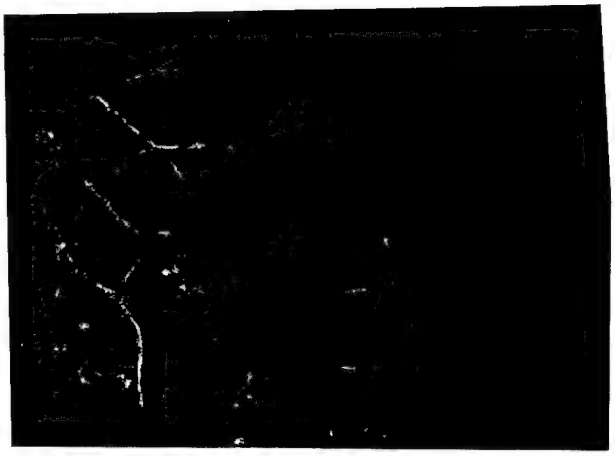
0 time



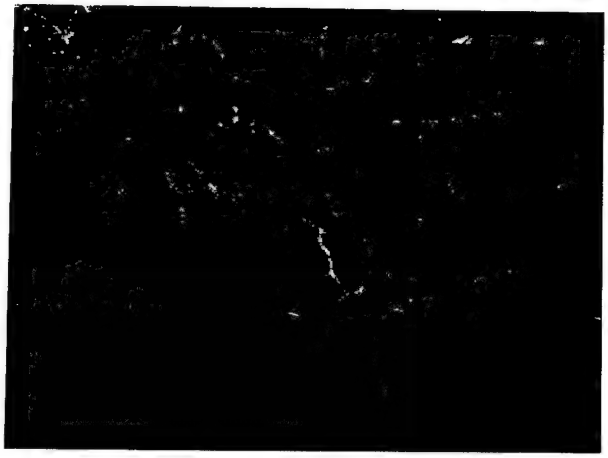
10 min



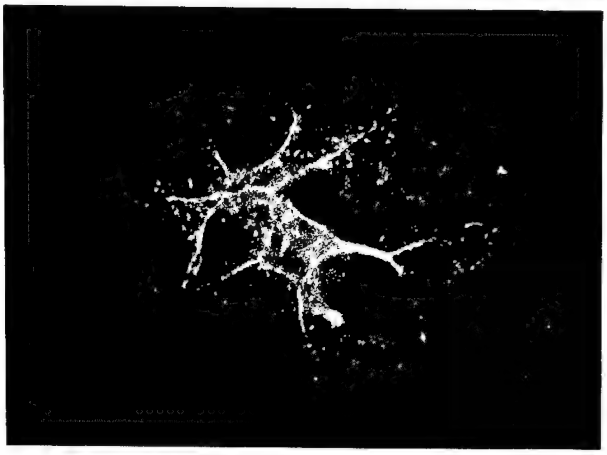
20 min



30 min

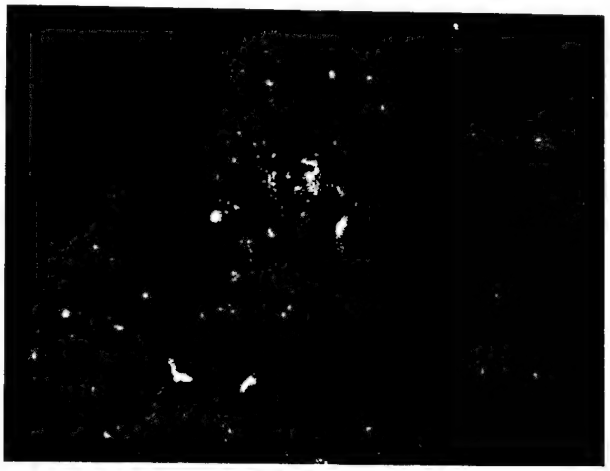


60 min



120 min

4h.



24 L.

Phospho specific antibody - Ab-18.

Figure 15. internalization of immunoconjugate in BT-474 cells. Cells were treated with immunoconjugate (1.5μg/ml) for indicated time and then treated with secondary antibody. Fluorescence was visualized under microscope.

FIGURE 15

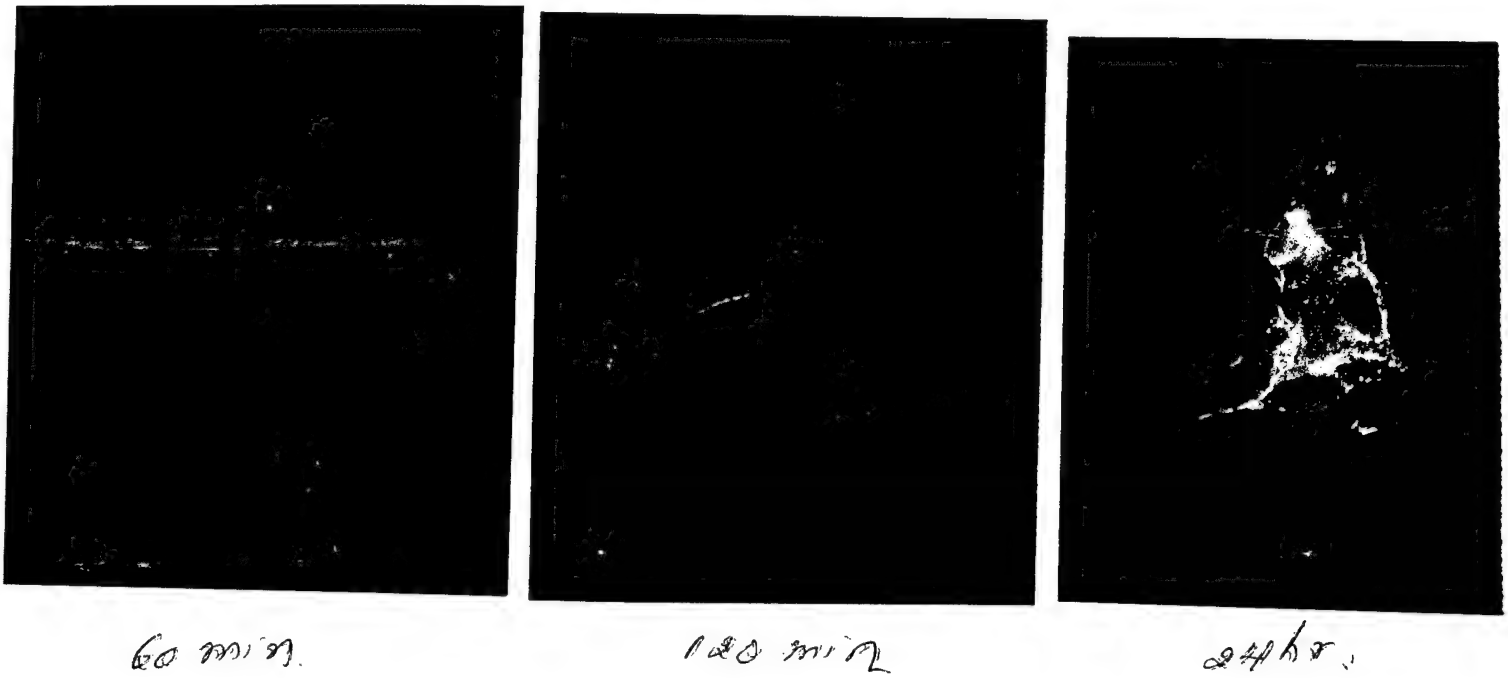
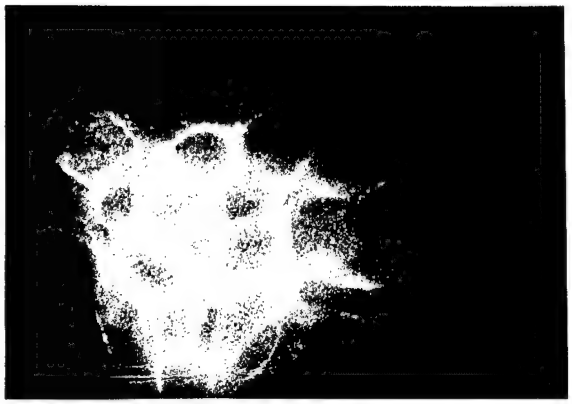


Figure 16. Total Her-2 expression in BT-474 cells following 7 days incubation in control culture medium (MEM-E containing 5% charcoal stripped serum), medium containing 10^{-7}M 1,25 $(OH)_2$ D_3 1μM 1α $(OH)D_5$, Her-2 antibody (1.5μg/ml) or Her-2 + 1α $(OH)D_5$. At the end of incubation cells were fixed in formalin and then incubated with FITC-labeled secondary antibody.

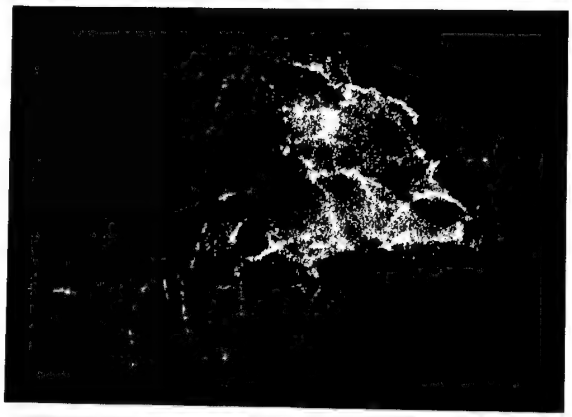
FIGURE 16 A

Control media
7day



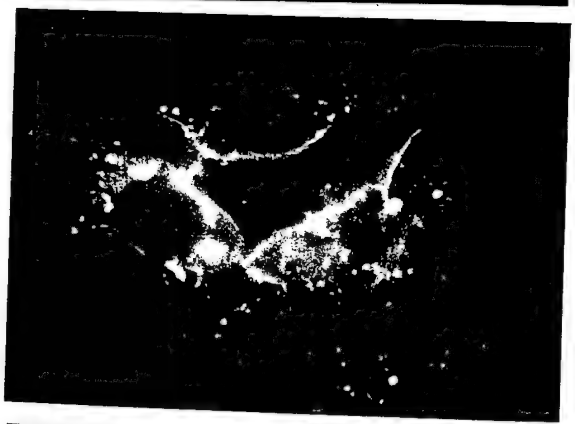
Total Sol. SS
Her2 receptor.

D_3 10m incubation
7days



D_5 10m incubation
7days

Her-2 incubation
15 ng/ml
7days
(7d)



Her-2 + D_5 incubation
7days

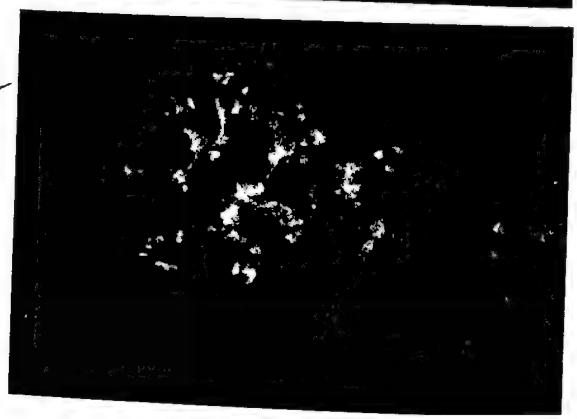


Figure 16b. Western blot analysis of Her-2 protein. BT-474 cells were treated with $1,25(OH)D_3$, $1\alpha(OH)D_5$, Her-2 antibody, D_5 +Her-2 antibody or $1\alpha(OH)D_5$-Her-2 immunoconjugate. Following incubation, cells were washed with PBS, cell lysates were prepared, and 50 μg of total protein was loaded on 4-20% polyacrylamide gel. After immunoblotting, membrane was incubated with Her-2 antibody, the secondary HRP-labeled antibody. Specific protein immunoreactivity was visualized by chemiluminescence.

FIGURE 16B

Control

D_3^-7

D_5^-6

Her-2-neu

D_5 Her -2-neu

Immuno-conjugate

Figure 17. Effect of immunoconjugate on in vivo growth of BT-474 cells. BT-474 cells were inoculated s.c. into female athymic mice. All animals received estradiol pellet. Animals were divided into the following groups: 1) receiving control regular diet; 2) receiving diet supplemented with 1α(OH)D_5 (12.5 μg/kg diet); 3) receiving i.p. administration of 5 μg Her-2 antibody once weekly; 4) receiving 1α(OH)D_5-supplemented diet and receiving antibody treatment as in group 3; 5) receiving i.p. administration of immunoconjugate equivalent to 5 μg Her-2 antibody. Tumor volume in each group was determined weekly. Data represent mean ± S.E.

FIGURE 17

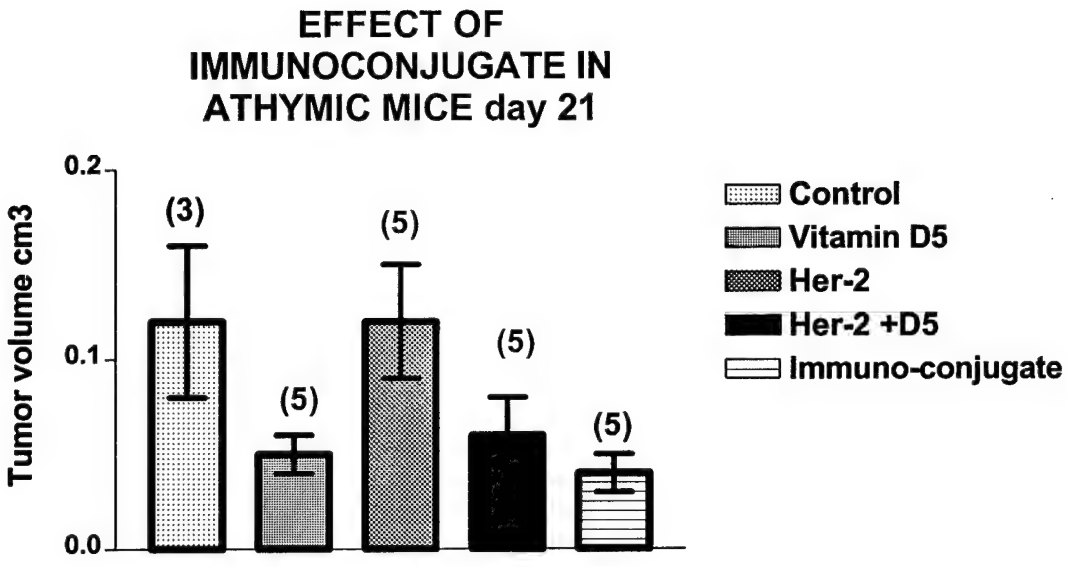

EFFECT OF
IMMUNOCONJUGATE IN
ATHYMIC MICE day 21
Tumor volume cm3
0.2
0.1
0.0
(3)
(5)
(5)
(5)
(5)
Control
Vitamin D5
Her-2
Her-2 +D5
Immuno-conjugate

Figure 18. Effect of immunoconjugate on growth of BT-474 cells transplanted into athymic mice. BT-474 cells were inoculated s.c. into female athymic mice. All animals received estradiol pellet. Animals were divided into the following groups: 1) receiving control regular diet; 2) receiving diet supplemented with $1\alpha(OH)D_5$ (12.5 μg/kg diet); 3) receiving i.p. administration of 5 μg Her-2 antibody once weekly; 4) receiving $1\alpha(OH)D_5$-supplemented diet and receiving antibody treatment as in group 3; 5) receiving i.p. administration of immunoconjugate equivalent to 5 μg Her-2 antibody. Tumor volume in each group was determined weekly. Data represent mean $\pm$ S.E.

FIGURE 18

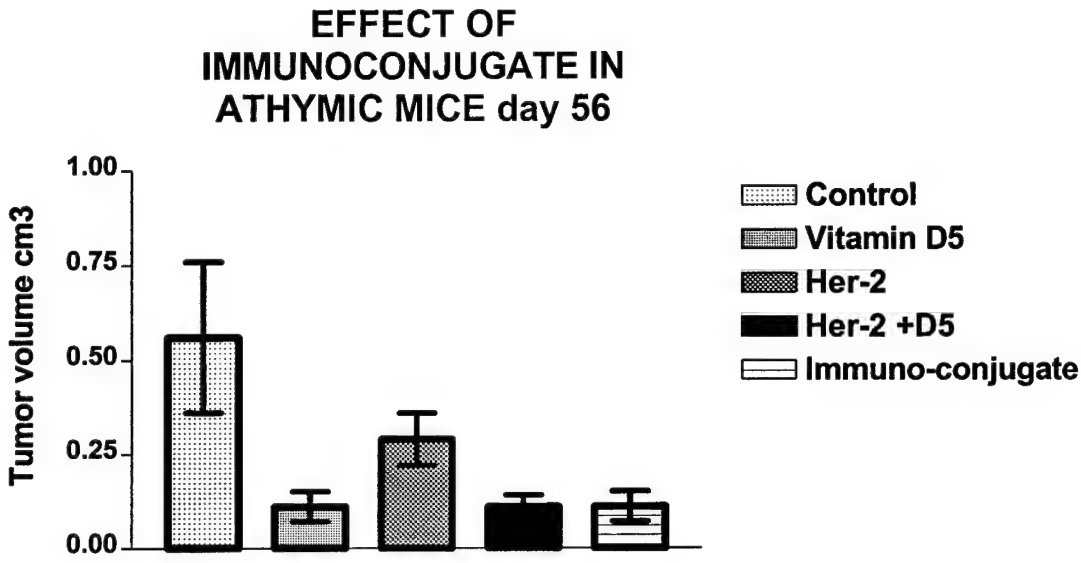

EFFECT OF
IMMUNOCONJUGATE IN
ATHYMIC MICE day 56
Tumor volume cm3
1.00
0.75
0.50
0.25
0.00
Control
Vitamin D5
Her-2
Her-2 +D5
Immuno-conjugate

Figure 19. Effect of $1\alpha(OH)D_5$ (12.5 μg/kg diet)-supplemented diet, Her-2 (5 μg/weekly) administration, and combination treatment with both on in vivo growth of BT-474 cells transplanted in female athymic mice. Data represent mean ± SE.

FIGURE 19

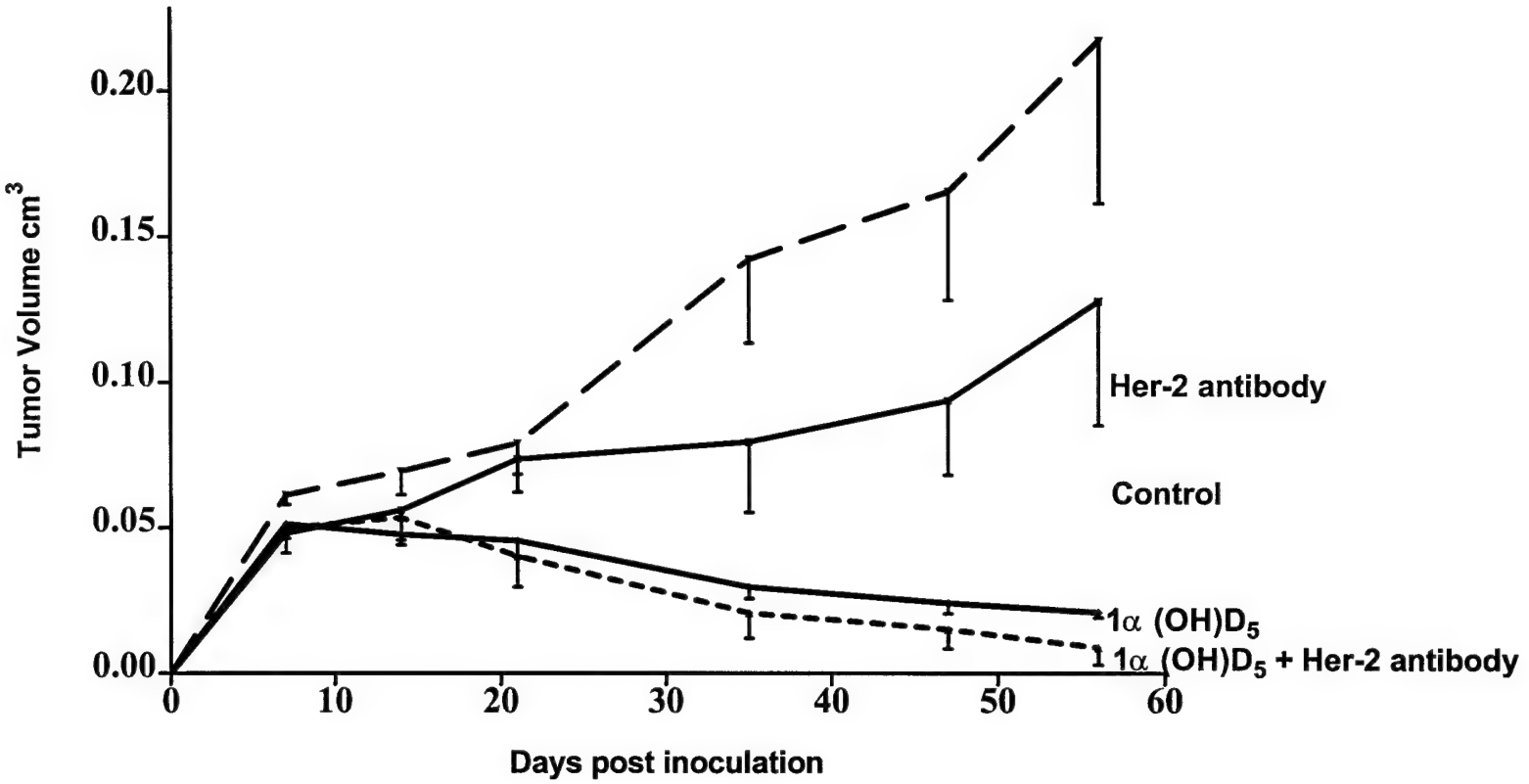

Tumor Volume cm3
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
50
60
Days post inoculation
Her-2 antibody
Control
1α (OH)D5
1α (OH)D5 + Her-2 antibody

Figure 20. Histology of BT-474 xenograft tumors originated in athymic mice receiving: 1) control diet; 2) diet supplemented with $1\alpha(OH)D_5$; 3) Her-2 antibody; or 4) Her-2 antibody + vitamin D analog.

FIGURE 20

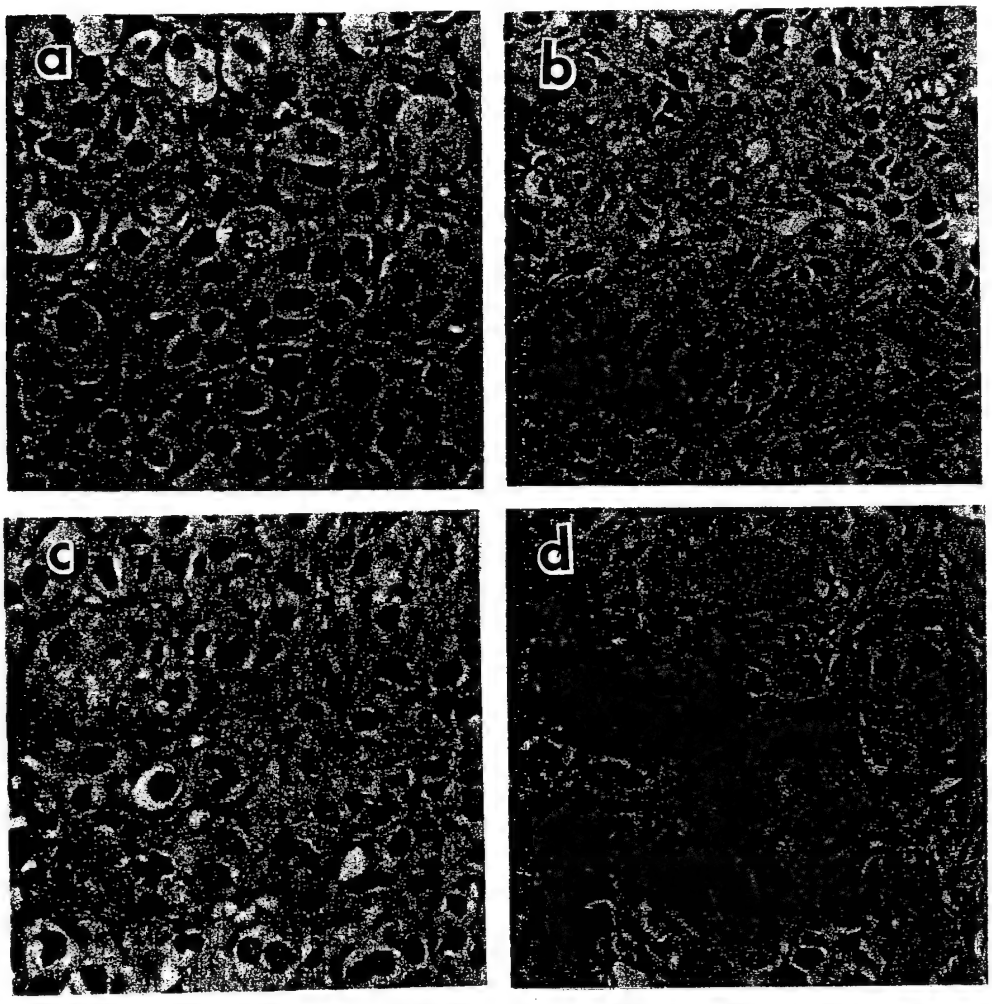


Figure 21. Effect of $1\alpha(OH)D_5$ supplemented in the diet, Her-2 administration, and combination treatment with $1\alpha(OH)D_5$ + Her-2 administration on in vivo growth of ZR-75-1 cells transplanted into female athymic mice. Data represent mean $\pm$ S.E.

FIGURE 21

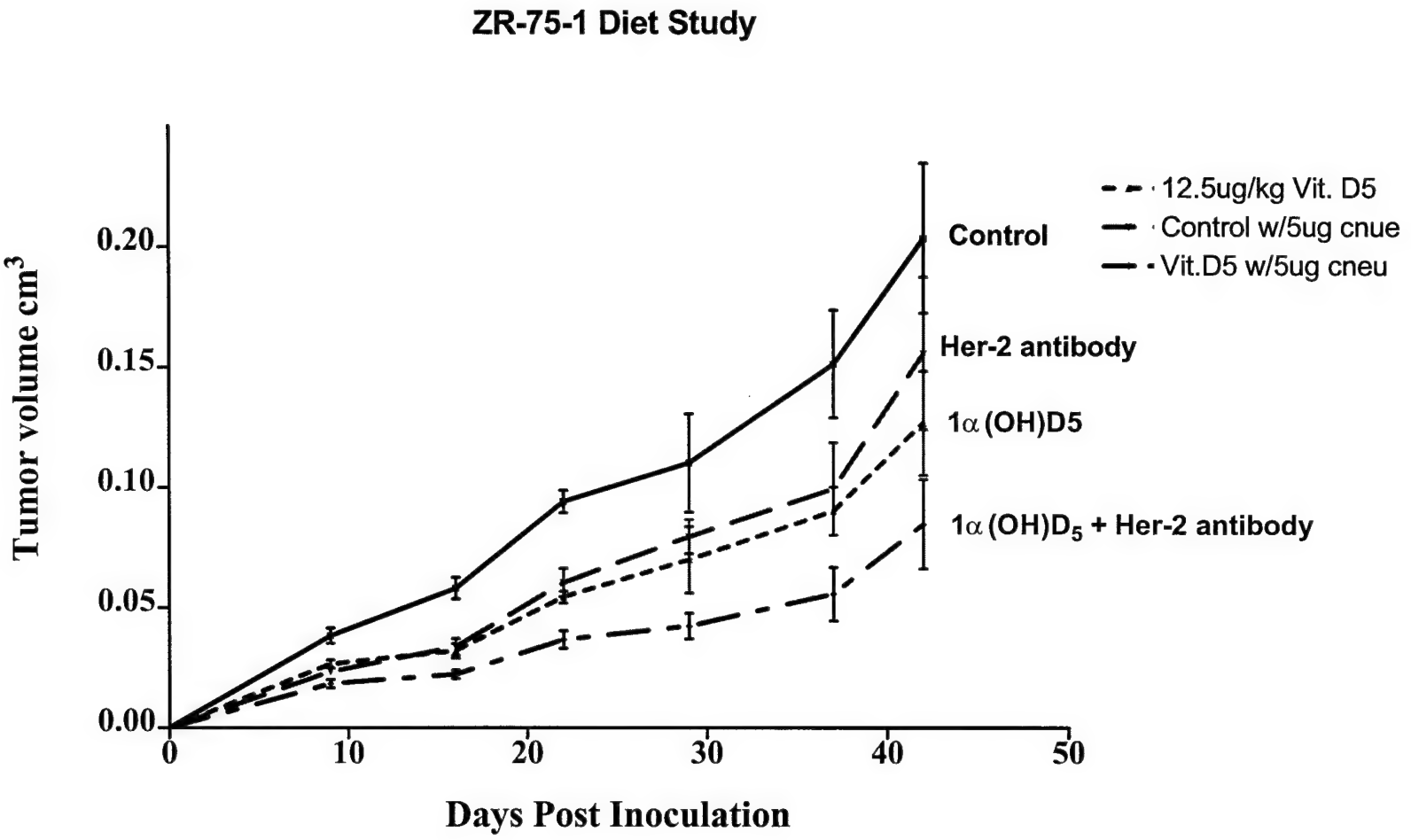

ZR-75-1 Diet Study
Tumor volume cm3
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
50
Days Post Inoculation
12.5ug/kg Vit. D5
Control w/5ug cnue
Vit.D5 w/5ug cneu
Control
Her-2 antibody
1α(OH)D5
1α(OH)D5 + Her-2 antibody

Figure 22. Histology of xenografts from animals in Figure 21 treated with control diet and 1α(OH)D_5-supplemented diet.

FIGURE 22

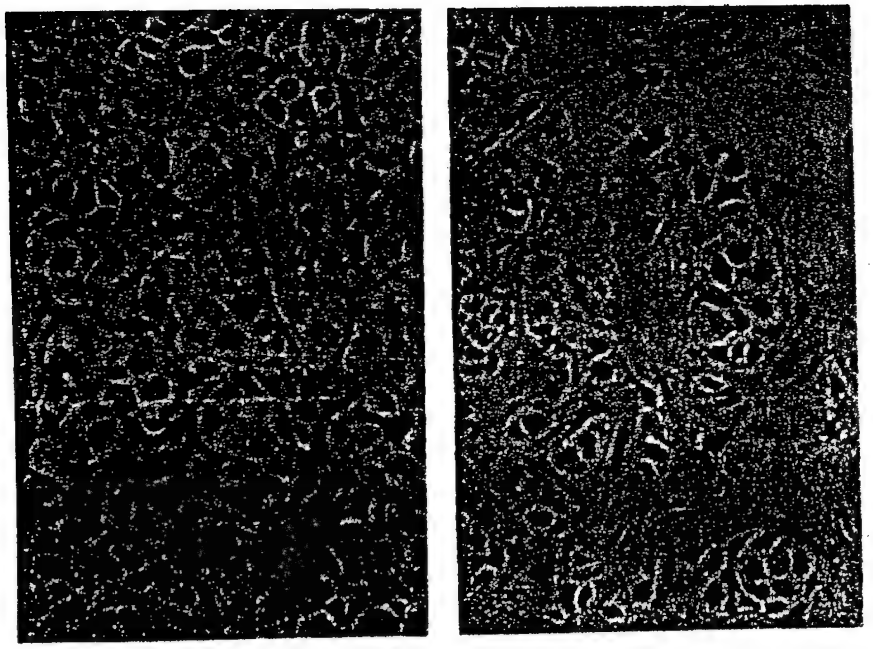


Figure 23. Growth of ZR-75-1 xenografts after discontinuation of $1\alpha(OH)D_5$ dietary supplementation and Her-2 antibody treatment. Data represent mean $\pm$ S.E.

FIGURE 23

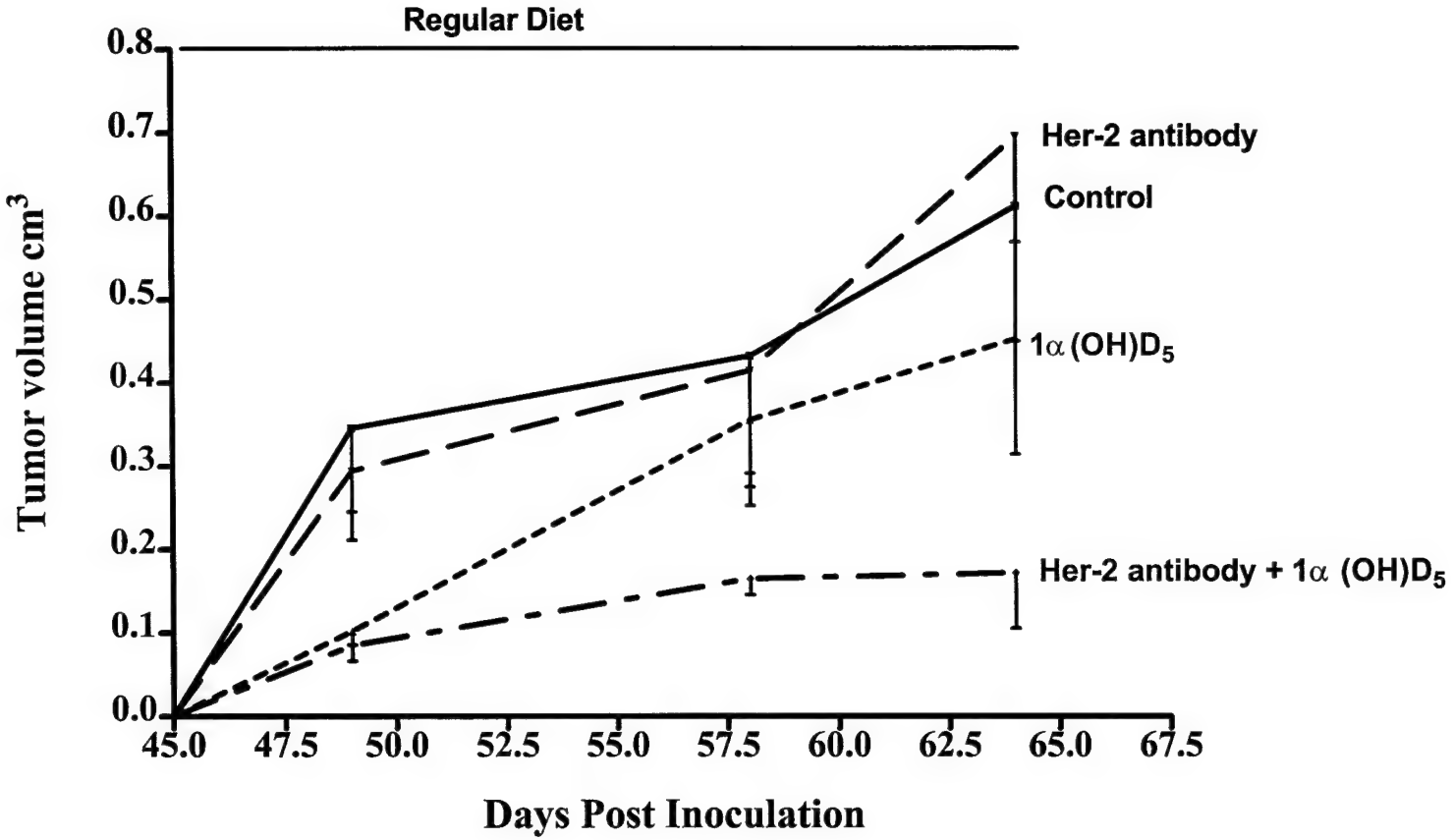

Regular Diet
Her-2 antibody
Control
1α (OH)D5
Her-2 antibody + 1α (OH)D5
Tumor volume cm3
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
45.0
47.5
50.0
52.5
55.0
57.5
60.0
62.5
65.0
67.5
Days Post Inoculation

Figure 24. Histology of ZR-75-1 xenografts at the time of termination. Animals received: a) control diet; b) diet supplemented with $1\alpha(OH)D_5$; c) Her-2 administration; or d) Her-2 + D5 treatment as in group 2. All animals received specified treatments for 45 days, and then all animals were switched to regular diet.

FIGURE 24

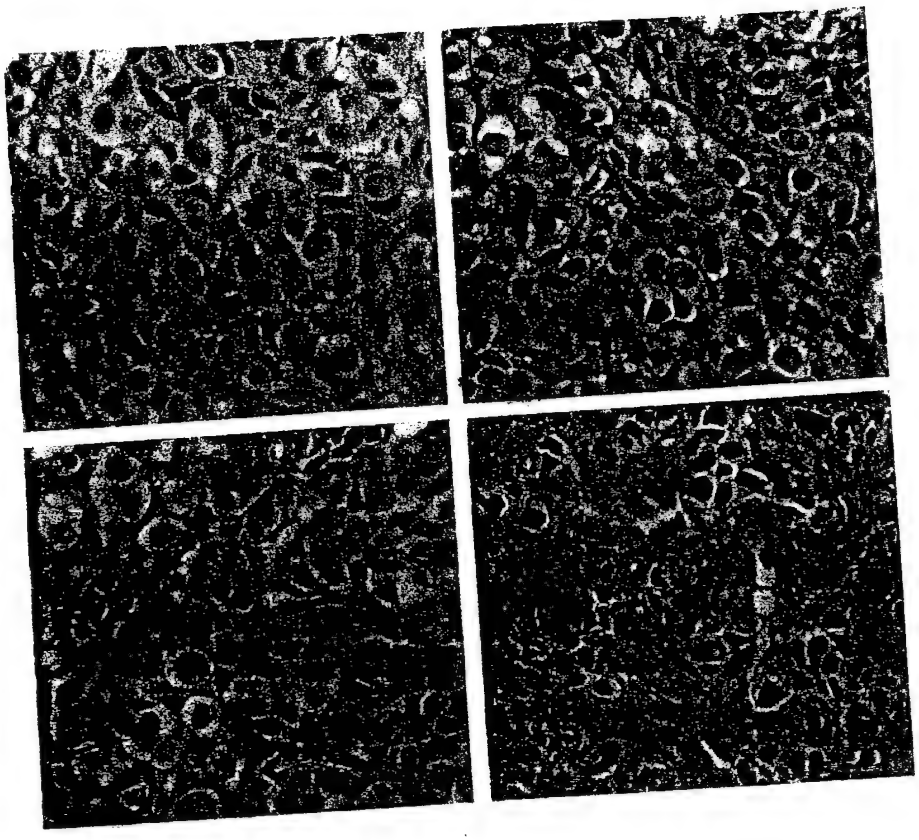


Figure 25. Serum calcium levels in animals receiving control diet, 1α(OH)D_5 supplemented diet, Her-2 antibody treatment, or Her-2 + 1α(OH)D_5 treatment.

FIGURE 25

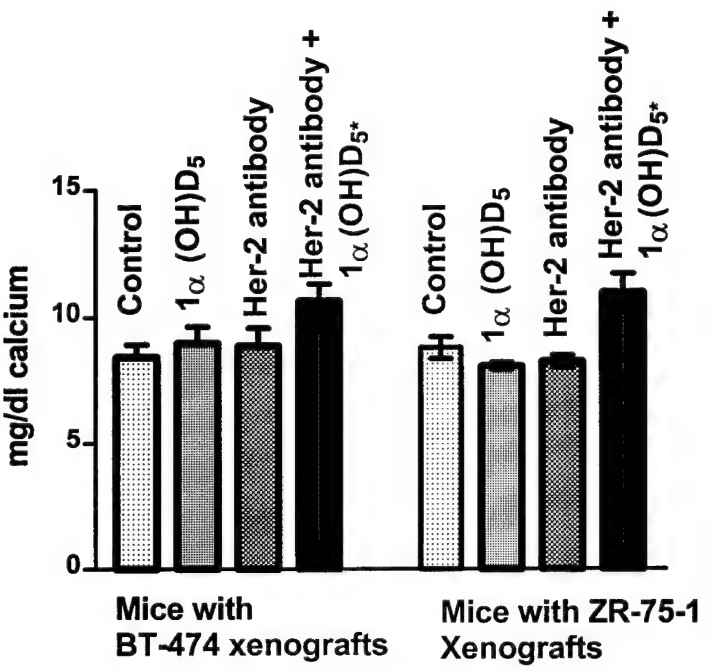

mg/dl calcium
15
10
5
0
Control
1α (OH)D5
Her-2 antibody
Her-2 antibody + 1α(OH)D5*
Control
1α (OH)D5
Her-2 antibody
Her-2 antibody + 1α(OH)D5*
Mice with BT-474 xenografts
Mice with ZR-75-1 Xenografts

Figure 26. Body weight in animals receiving control diet, diet supplemented with $1\alpha(OH)D_5$, Her-2 antibody, or Her-2 antibody + $1\alpha(OH)D_5$. Body weights are shown as mean$\pm$S.E. in each group. Body weights are also shown after discontinuation of treatments.

FIGURE 26

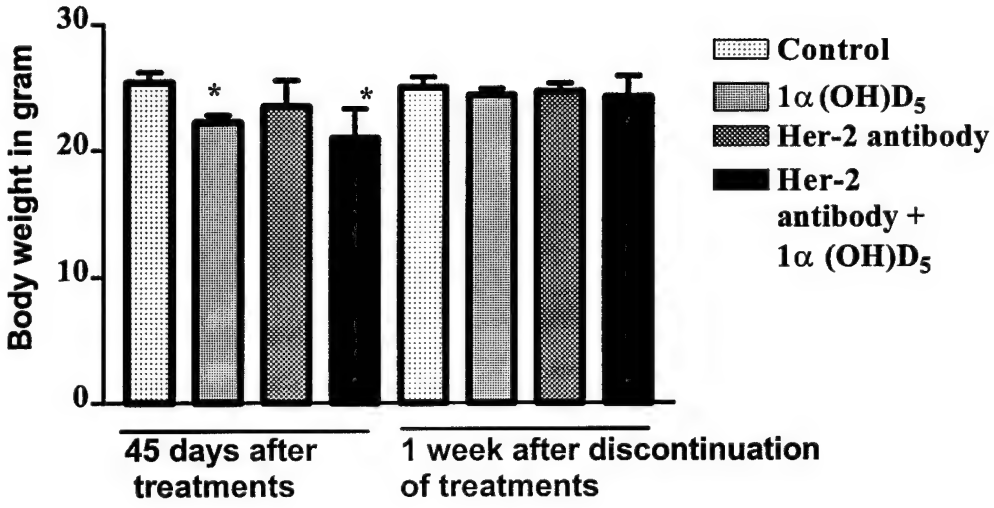
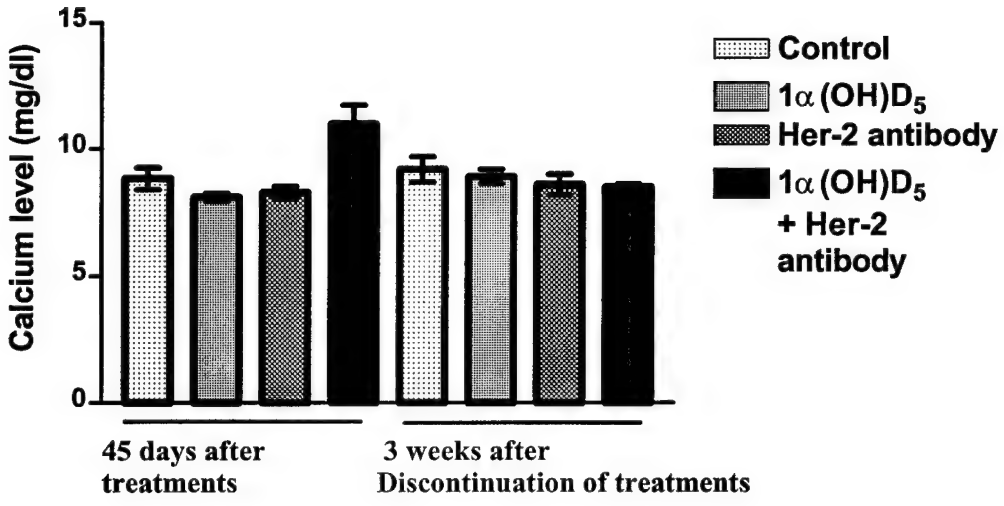

Body weight in gram
30
20
10
0
*
*
Control
1α (OH)D5
Her-2 antibody
Her-2 antibody + 1α (OH)D5
45 days after treatments
1 week after discontinuation of treatments

Figure 27. Serum calcium levels in animals bearing ZR-75-1 xenograft after 45 days treatment and 3 weeks after discontinuation of treatment. Data represent mean + S.E.

FIGURE 27


15
10
5
0
Calcium level (mg/dl)
Control
1α (OH)D5
Her-2 antibody
1α (OH)D5
+ Her-2
antibody
45 days after
treatments
3 weeks after
Discontinuation of treatments

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Abstract

The role of the active metabolite of vitamin D, 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), in cell differentiation is well established. However, its use as a differentiating agent in a clinical setting is precluded due to its hypercalcaemic activity. Recently, we synthesised a relatively non-calcaemic analogue of vitamin D_5, 1α-hydroxyvitamin D_5 ($1\alpha(OH)D_5$), which inhibited the development of carcinogen-induced mammary lesions in culture and suppressed the incidence of chemically induced mammary carcinogmas in rats. In the present study, we determined the differentiating effects of 1α-$(OH)D_5$ in T47D human breast cancer cells and compared its effects with $1,25(OH)_2D_3$. Cells incubated with either 10 or 100 nM of the analogues inhibited cell proliferation in a dose-dependent manner, as measured by the dimethylthiazolyl-2,5-diphenyltetrazolium bromide (MTT) assay. Similar growth-inhibitory effects were also observed for $MCF10_{neo}$ cells. Both vitamin D analogues induced cell differentiation, as determined by induction of casein expression and lipid production. However, $MCF10_{neo}$ cells failed to respond to either vitamin D analogue and did not undergo cell differentiation. Since the cell differentiating effect of vitamin D is considered to be mediated via the vitamin D receptor (VDR), we examined the induction of VDR using reverse transcriptase–polymerase chain reaction (RT-PCR) in both cells. The results showed that, in T47D cells, both $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ induced VDR in a dose-dependent manner. Moreover, both analogues of vitamin D upregulated the expression of vitamin D response element-chloramphenicol acetyl transferase (VDRE-CAT). These results collectively indicate that 1α-$(OH)D_5$ may mediate its cell-differentiating action via VDR in a manner similar to that of $1,25(OH)_2D_3$. © 2000 Published by Elsevier Science Ltd. All rights reserved.

Keywords: Vitamin D; Breast cancer cells; Differentiation; T47D; MCF10

1. Introduction

The research on vitamin D_3 and related compounds is currently at its apex. A vast amount of evidence has been collected, implicating the essential involvement of vitamin D metabolites in several cellular processes. The active metabolite 1,25 dihydroxy vitamin D_3 ($1,25(OH)_2D_3$) and related compounds suppress the development and progression of breast cancer and other carcinomas *in vivo* [1,2], inhibit the metastatic spread of tumour cells [3–5], and promote differentiation of breast cancer cells [6–8]. However, the calcaemic side-effects of $1,25(OH)_2D_3$ have prevented its application as a pharmaceutical agent. In recent years, considerable attention has been given to the development of vitamin D_3 analogues capable of inducing cell differentiation without systemic hypercalcaemia [8–10]. Many structural modifications are known to enhance several-fold the differentiating potency of vitamin D_3 analogues in normal (usually keratinocyte) or malignant (usually leukaemia) cell lines. Little attempt, however, has been made to evaluate vitamin D analogues of other series such as vitamin D_2, D_4, D_5 and D_6. This structural classification is based on the differences encountered in the side chain. Earlier studies reported that vitamin D_5 was the least toxic of vitamins D_2 through to D_6 [11].

During the past 2 years, we have been studying the role of 1α-hydroxyvitamin D_5 ($1\alpha(OH)D_5$), an analogue of vitamin D_5 (24-ethyl-vitamin D_3), on breast cancer cell differentiation. We have characterised its calcaemic

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activity in vitamin D-deficient Sprague–Dawley rats [12]. The analogue $1\alpha(OH)D_5$ was synthesised from sitosterol acetate and was found to be less calcaemic than vitamin D_3. It was observed that $1\alpha(OH)D_5$ was effective against the development of carcinogen-induced mammary lesions in mouse mammary gland organ cultures [12]. In a more recent study, we observed that $1\alpha(OH)D_5$ inhibited incidence and tumour multiplicity of N-methyl-N-nitrosourea-induced mammary adenocarcinoma in rats (data not shown). These results clearly demonstrate that this vitamin D analogue might be a good candidate in the prevention of mammary carcinogenesis. In the present study, we evaluated the effects of $1\alpha(OH)D_5$ on cell differentiation and proliferation in oestrogen receptor (ER)-positive T47D breast cancer cells and compared the effects of the D_5 analogue with the active metabolite of vitamin D_3, $1,25(OH)_2D_3$. Moreover, we compared the effects of vitamin D analogues between ER+ T47D cells and ER- $MCF10_{neo}$ cells. Both cell lines are negative for functional p53 [13].

It is well known that the nuclear activity of vitamin D_3 is based on the interaction of the vitamin D active metabolite, $1,25(OH)_2D_3$, with the vitamin D receptor (VDR) [14]. The VDR is a nuclear receptor that belongs to the superfamily of ligand-dependent transcription factors and is expressed in all the vitamin D target tissues. VDR mediates its action by conjugating with the Retinoid X Receptor (RXR) [15–17]. The VDR-RXR dimer, once formed, is capable of recognising the vitamin D response element (VDRE) in the promoter region of the gene. The VDRE is composed of direct repeats of 6 DNA bases separated by 3-base intervening sequences [18]. Vitamin D appears to play an important role in stabilising and transactivating the VDR/RXR–VDRE complex [19,20]. Its interaction with VDR, therefore, represents the central step in the transmission of a signal to the transcription machinery, resulting in activation or suppression of transcription of genes leading ultimately to differentiation. We recently showed that the normal human breast epithelial cells lacking functional VDR do not respond to vitamin D to induce cell differentiation. However, transient transfection of VDR in these HBL-100 cells resulted in increased association of VDR-VDRE, as measured by the CAT reporter assay [21]. In the present study, we compared the effects of $1\alpha(OH)D_5$ and $1,25(OH)_2D_3$ on the transactivation of VDR–VDRE in T47D cells.

2. Materials and methods

2.1. Cells

The breast epithelial cell line, $MCF10_{neo}$, and human breast cancer cell line, T47D, were obtained from the American Type Culture Collection (Rockville, MD, USA). The $MCF10_{neo}$ cells were maintained in minimum essential medium with Earl's salts (MEME) medium supplemented with 10% fetal bovine serum (FBS), whereas T47D cells were maintained in RPMI supplemented with 0.2 I.U. bovine insulin/ml and 10% FBS. The monkey renal cancer CV-1 cells were maintained in MEME with 10% FBS supplement.

2.2. MTT assay

The cells were seeded in a 96-well/plate at a density of 500 cells/well in 100 μl/well of cell culture medium supplemented with 10% steroid-stripped serum. 24 h after seeding, the cells were incubated with 10 and 100 nM concentrations of $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$, respectively. The medium was changed every 3 days. After 7 days, the cultures were used for the dimethylthiazolyl-2,5-diphenyltetrazolium Bromide (MTT) assay. MTT (5 mg/ml in phosphate buffered serum (PBS)) was added to the wells (15 μl/well) and incubated at 37°C for 2 h. The stop solution (20% sodium dodecyl sulphate (SDS) in 50% N,N-dimethylformamide) was then added (100 μl/well) and incubated for an additional 2 h. The plates were scanned at 590 nm OD, and the results for each treatment group were averaged.

2.3. Immunohistochemistry

$MCF10_{neo}$ and T47D cells were grown to subconfluence on coverslips for 7 days, washed in PBS and fixed in 10% formalin for 5 min. The fixed cells were further incubated in cold methanol for 3 min and acetone for 2 min. After blocking the cells with a protein block/normal goat serum (BioGenex, San Ramos, CA, USA), they were incubated with casein antibody (100 μg/ml) (Accurate Chemical and Scientific Corp. Westbury, NY, USA) for 2 h. The cells were then incubated with secondary anti-mouse biotinylated antibody for 30 min, followed by streptavidin–peroxidase complex and 3,3′-diaminobenzidine (DAB) solution as chromogen. Appropriate controls were performed to rule out non-specific staining with secondary antibody.

2.4. Lipid assay

$MCF10_{neo}$ and T47D cells were grown to subconfluence on coverslips for 7 days, washed in PBS, and fixed by incubating in cold methanol for 3 min and propylene glycol for 2 min. The cells were, at this point, stained with Oil Red O' for 30 min and rinsed in isopropyl alcohol then de-ionised water. Haematoxylene staining for 30 s and Scott solution rinse completed the assay.

2.5. RNA isolation and RT-PCR

The cells were incubated with 1, 10 or 100 nM $1,25(OH)_2D_3$ or $1\alpha(OH)D_5$ for 3 days. The medium

from the tissue culture flasks was removed and the cells were treated with RNA-Zol B (Tel-Test Inc., Friendswood, TX, USA). RNA were isolated according to the manufacturer's instruction and quantified spectrophotometrically. Reverse transcription (RT) and PCR were carried out using Advantage RT for PCR and Advantage cDNA PCR kit (Clontech Inc., Palo Alto, CA, USA). Primer sequences for VDR were selected and custom-synthesised by Oligos Etc. The sense primer was 5′-GGA GTT GCT GTT TGT TTG AC, and the antisense primer was 5′-CTT CTG TGA GGC TGT TTT TG. The primer for the housekeeping gene *G3PDH* was purchased from ClonTech. The touchdown PCR procedure was employed with minor modifications [22]. The first strand cDNA was heated at 94°C for 1 min followed by denaturation at 94°C for 45 sec, annealing at 68–66–64–62–60°C for 45 sec each time and extension at 72°C for 2 min, for 26 cycles. The final cycle was followed by a 7-min extension step at 72°C to ensure that the amplified DNA was double stranded. The absence of contaminant was routinely checked by RT-PCR assays of negative control samples (sterile buffer, provided in the kit). The PCR products were separated on 1.5% agarose gel at 64 volts for 3 h, stained with ethidium bromide and visualised by ultraviolet (UV)-transillumination.

2.6. Transient transfection

The reporter construct VDRE-tk-CAT was prepared by inserting a copy of VDRE into the *Bam*HI site of the $pBLCAT_2$ as previously described [23]. For transfection, 1×10^5 CV-1 cells were plated in 24-well plates. Transfections were carried out using the calcium phosphate precipitation procedure. Briefly, 100 ng of VDRE-tk-CAT reporter plasmid, 250 ng of β-galactosidase (β-gal) expression vector, and 500 ng of VDR expression vectors were mixed with carrier DNA (pBluescript) to 1 μg of total DNA per well. The CAT activity was normalised for transfection efficiency by the corresponding β-gal activity.

3. Results

3.1. Effect of $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ on cell proliferation

The breast epithelial cells $MCF10_{neo}$ and breast cancer cells T47D were incubated with the vitamin D analogues for 7 days in culture. After this, the effects of vitamin D analogues were evaluated by the MTT assay. The results indicated a 31% and 50% growth inhibition for $MCF10_{neo}$ at 10 and 100 nM of $1,25(OH)_2D_3$ concentrations, respectively, as compared with 50% and 72% inhibition with $1\alpha(OH)D_5$ at 10 and 100 nM, respectively (Fig. 1a). The ER-positive, T47D cells showed a 29% and 52.5% growth inhibition after being exposed for 7 days to $1,25(OH)_2D_3$ at 10 and 100 nM, respectively. Unlike $MCF10_{neo}$ cells, T47D cells did not exhibit increased growth suppression when exposed to $1\alpha(OH)D_5$. Both analogues suppressed growth of T47D cells by approximately 30% and 50% at low and high concentrations, respectively (Fig. 1b). These results suggest that both $1\alpha(OH)D_5$ and $1,25(OH)_2D_3$ are comparable in producing antiproliferative effects in breast cancer cells.

3.2. Induction of differentiation of breast cancer cell lines

Since one of the major recognised functions of vitamin D is induction of cell differentiation, we evaluated the effects of both analogues on the induction of differentiation in both cell lines. As markers of cell differentiation, we used casein and lipid. Casein expression was measured by immunocytochemistry using casein antibodies. Results showed that, for T47D cells, casein was expressed in less than 10% of the control cells.

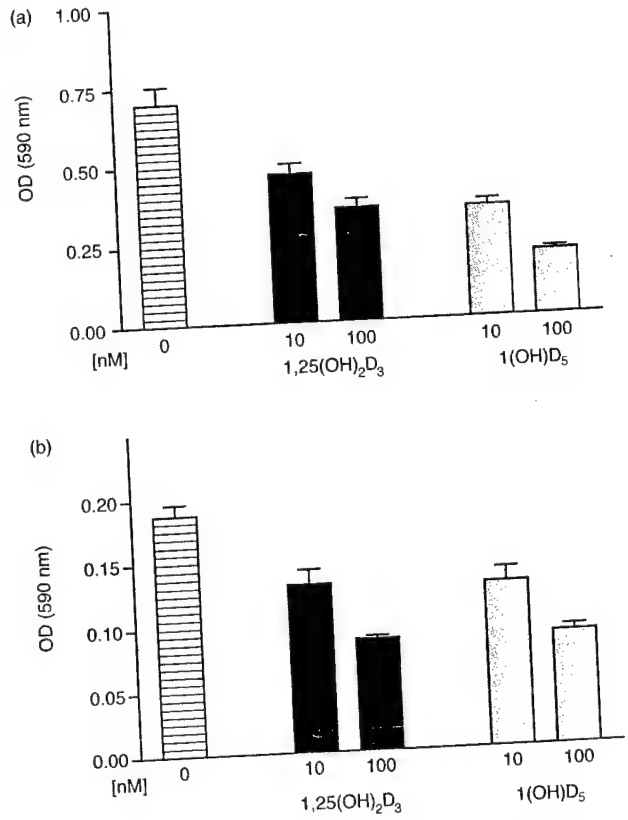

Fig. 1. Effects of $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ on the proliferation of $MCF10_{neo}$ cells and T47D cells. The MTT assay was carried out using duplicate cultures and the experiments were repeated three times. The error bars represent the standard deviation. (a) $MCF10_{neo}$ cells; (b) T47D cells.

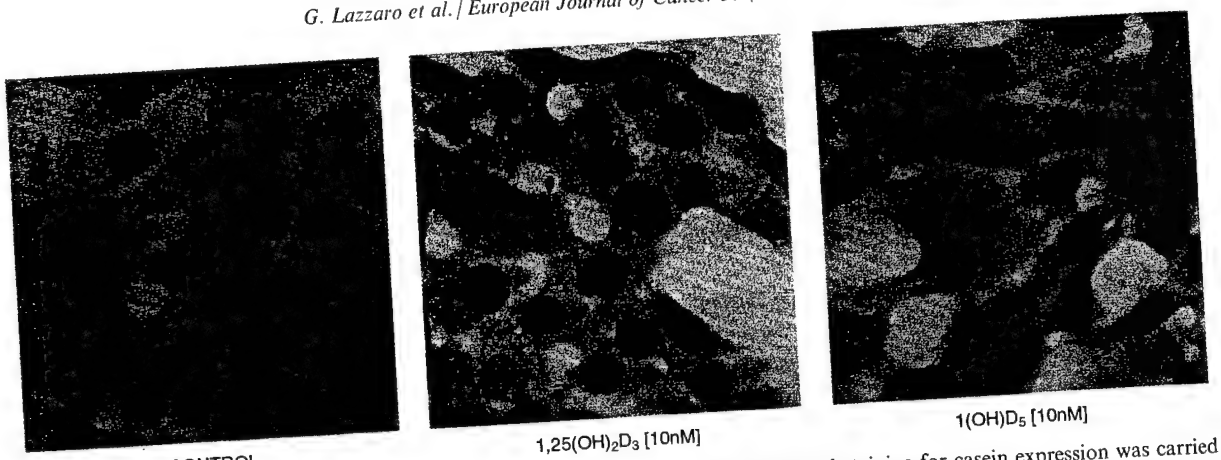

Fig. 2. Effect of vitamin D analogues on casein expression in T47D cells. Immunohistochemical staining for casein expression was carried out as previously described in the presence or absence of the vitamin D analogues.

After 7 days treatment with $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$, the intensity and number of cells expressing casein increased to approximately 70 and 85% at 10 and 100 nM concentrations, respectively. No difference was noticed between the effects of D_3 or D_5 analogues (Fig. 2 and data not shown). Similarly, there was a dramatic increase in the expression of lipid production in T47D cells after 7 days of treatment with $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ (Fig. 3). These results indicated that both vitamin D analogues induce cell differentiation in T47D cells. In contrast, the $MCF10_{neo}$ cells, tested for the same markers of differentiation, did not show any presence or induction of either casein or lipids in the control cells or in cells exposed to vitamin D_3 or D_5 (data not shown).

3.3. Transactivation of VDRE

The VDRE transactivation activity of the vitamin D analogues was determined using the *CAT* reporter gene containing VDRE (VDRE-tk-CAT). In order to compare the activity of $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ for transactivating the *VDRE* reporter gene, we selected monkey renal cancer cells (CV-1). These cells lack a functional VDR, so one can evaluate the binding activity of vitamin D analogues only in the transiently transfected VDR. The active metabolite of vitamin D, $1,25(OH)_2D_3$, should not show any increase in CAT activity if the cells are transfected only with VDRE-tk-CAT. As shown in Fig. 4, neither vitamin D_3 nor vitamin D_5 analogues could induce CAT activity, indicating a lack of endogenous VDR in these cells. However, when 500 ng VDR (Fig. 4b) was co-transfected with VDRE and the cells were incubated with 10 or 100 nM of $1,25(OH)_2D_3$ or $1\alpha(OH)D_5$, there was enhanced expression of the *CAT* reporter gene. These results clearly indicate that both analogues of vitamin D can bind to the VDR and the complex can bind to the VDRE to initiate signal transduction. However, the extent of VDRE-reporter transactivation was 7- to 8-fold greater when the transfected cells were incubated with $1,25(OH)_2D_3$ at 10 nM and nearly 2-fold greater at 100 nM, respectively, compared with $1\alpha(OH)D_5$ at the same concentrations. This is consistent with the observed

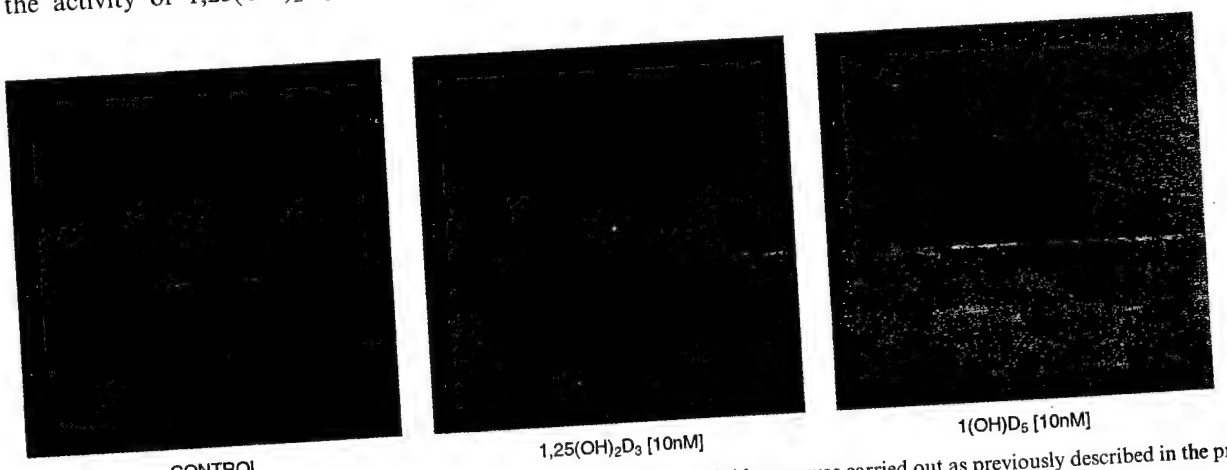

Fig. 3. Effects of $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ on lipid expression in T47D cells. A lipid assay was carried out as previously described in the presence or absence of vitamin D analogues.

finding that a log molar higher concentration of $1(OH)D_5$ is needed to obtain an equivalent response to that observed with $1,25(OH)_2D_3$.

3.4. Induction of VDR mRNA as determined by RT-PCR

Experiments were carried out to determine if VDR mRNA is induced by the vitamin D analogues in T47D and $MCF10_{neo}$ cells. Total RNA from the cells was isolated and reverse-transcribed. The cDNA was amplified using Taq polymerase and separated on 1.5% agarose gel. As shown in Fig. 5, the housekeeping gene *G3PDH* (C) was identical for all the cDNAs, indicating an equal loading of the gels. The VDR separated as a 420 bp fragment on the gel. As shown in Fig. 5(a), in T47D cells, there was a basal level of expression of VDR; however, incubation of cells for 3 days with either 10 or 100 nM of $1,25(OH)_2D_3$ increased the VDR expression in a dose-related manner. Similar results were also obtained with $1\alpha(OH)D_5$, as shown in Fig. 5(a). In contrast, $MCF10_{neo}$ cells expressed the basal level of VDR in the cells; but, there was no induction of VDR message by the vitamin D analogues (Fig. 5b). These results indicate that the lack of induction of differentiation by vitamin D in $MCF10_{neo}$ cells may be related to a lack of induction of VDR in these cells by vitamin D analogues.

4. Discussion

The effects of vitamin D analogues as differentiating agents and inhibitors of cell proliferation for breast cancer cells have been reported [1,7]. It is generally believed that the cells expressing VDR often respond to vitamin D analogues, whereas cells such as MDA-MB-231, which are ER- and express low or non-detectable

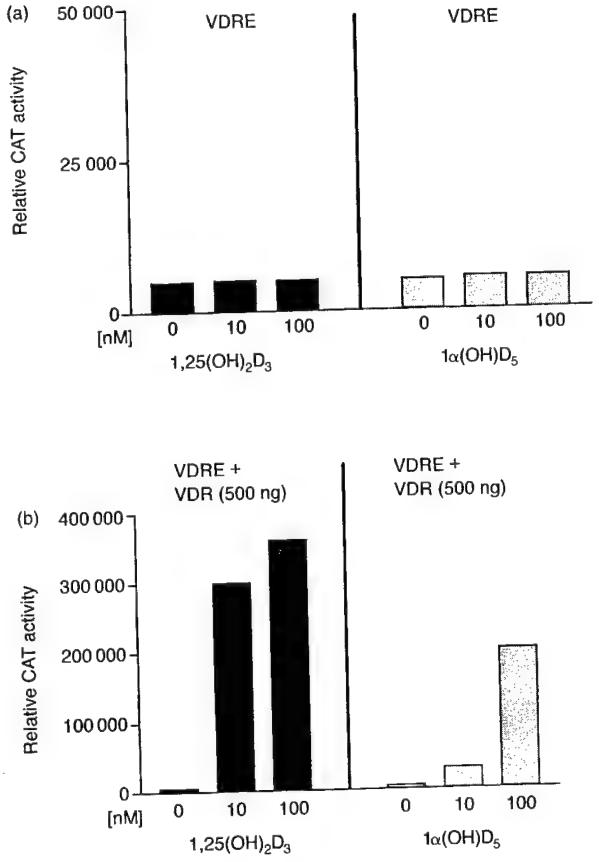

Fig. 4. Effects of $1,25(OH)D_3$ and $1\alpha(OH)D_5$ on the transactivation of the VDRE-conjugated reporter gene. Transient transfections of CV-1 cells with either VDRE-tk-CAT alone (a) or with VDR (b) was carried out by the calcium phosphate precipitation procedure. The cells were incubated with 10 and 100 nM vitamin D analogues for 3 days. CAT activity was measured spectrophotometrically. The experiments were repeated twice.

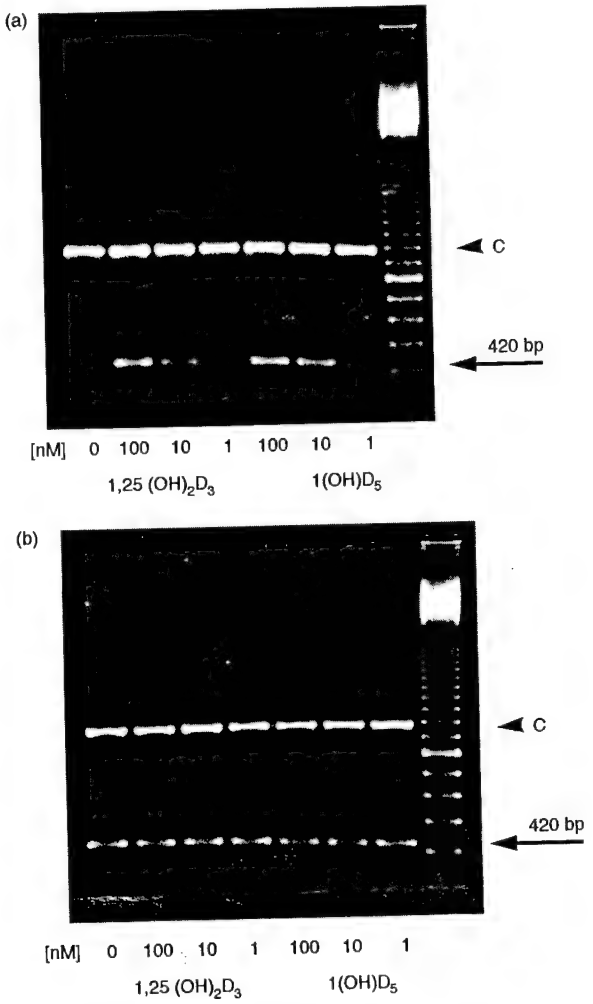

Fig. 5. Effects of vitamin D analogues on the expression of VDR mRNA in $MCF10_{neo}$ and T47D cells. Cells were incubated with various concentrations of analogues for 3 days in culture as previously described. VDR expression was measured by RT-PCR in (a) T47D cells; (b) $MCF10_{neo}$ cells. C, control housekeeping gene.

levels of VDR, do not respond to active vitamin D analogue(s) [24]. The VDR-mediated transcription regulatory genes include *TGFβ*, *EGF*, *c-myc* [25,26], and cell cycle regulators. The effects of various vitamin D analogues on programmed cell death have been evaluated in a variety of breast cancer cell lines. Consistently, MCF-7 cells which are ER+, VDR+ and positive for wild-type p53 exhibit apoptosis in response to vitamin D [27,28]. Although considerable literature exists for vitamin D-induced differentiation, its clinical application has been limited. This is due to its cytotoxicity at the concentration that induces differentiation. To this end, we have identified an analogue of the vitamin D_5 series which is non-calcaemic at the concentration at which 1,25-dihydroxyvitamin D_3 would induce hypercalcaemia. We previously reported that 1α-hydroxyvitamin D_5 inhibits carcinogen-induced development of mammary lesions in culture [12]. We also reported that it induces VDR and TGFβ in mammary epithelial cells. In this report, we addressed the question, "Does 1-hydroxyvitamin D_5 induce cell differentiation of breast cancer cells to the same extent as the active metabolite of vitamin D, 1,25-dihydroxyvitamin D_3?". T47D and $MCF10_{neo}$ cells were selected for the present study, since T47D cells are ER- and progesterone (PR)-positive and $MCF10_{neo}$ cells are negative for both ER and PR. Both analogues of vitamin D, $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$, inhibited cell proliferation to the same extent and induced differentiation as determined by the increased expression of differentiation markers.

The $MCF10_{neo}$ cells were originally derived from normal breast tissue and the epithelial cells were subsequently immortalised. The $MCF10_{neo}$ cells are ER-VDR+ and stably transfected with ras. The cells are tumorigenic in athymic mice. Since both T47D and $MCF10_{neo}$ have similar VDR and p53 status and differ only in their ER status, we compared the response of T47D ER+ and $MCF10_{neo}$ cells to two analogues of vitamin D. The $MCF10_{neo}$ cells, like T47D cells, exhibited a suppression of cell proliferation; however, no induction of differentiation was noticed. This, therefore, raised the question of whether induction of VDR is essential for cell differentiation. We evaluated the induction of VDR mRNA by these two vitamin D analogues. The results showed that $MCF10_{neo}$ cells constitutively expressed VDR-mRNA. However, there was no induction of the VDR message by either of the vitamin D analogues. In contrast, there was a dose-dependent increase in the expression of VDR mRNA in the T47D cells by both vitamin D_3 and D_5 analogues. These results suggest that there may be a positive association between the differentiation of cells by vitamin D and the induction of vitamin D-induced mRNA of VDR. Alternatively, the antiproliferative effects may be mediated by p53 although this is most unlikely in this case as both $MCF10_{neo}$ and T47D cells do not have functional p53 [10] and yet they respond to antiproliferative activity of vitamin D analogues. These results suggest that the antiproliferative effects and differentiating effects of vitamin D analogues may be independent of the cellular p53 status. These results are consistent with a recent report indicating the non-involvement of p53 in vitamin D-mediated differentiating/cell growth suppressing functions in breast cancer cells. Thus, it is not clear what mechanism may be operative for the suppression of cell growth by vitamin D analogues. If both antiproliferative effects and cell differentiating effects are mediated by VDR, then it is possible that the constitutive level of VDR will be sufficient to mediate vitamin D's effects in suppressing cell proliferation but that induction of new VDR mRNA may be necessary for cell differentiation.

Comparison between the action of a natural ligand of vitamin D, $1,25(OH_2)D_3$, and a vitamin D_5 analogue was also made in terms of their ability to transactivate a VDRE-reporter *CAT* gene. We selected VDR-negative CV-1 cells for these studies so that the endogenous VDR would not interfere with the interpretation of data. Since CV-1 cells are truly VDR-negative, they do not respond to incubation with $1,25(OH_2)D_3$ and do not transactivate the VDRE-CAT reporter. Since both T47D and $MCF10_{neo}$ cells express basal levels, to different extents, of VDR, the vitamin D analogue-induced transactivation of the CAT reporter may vary between these two cells and will compromise comparing the two analogues of vitamin D. Results showed that both $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ bind VDR and interact with VDRE. It was noted that, with 500 ng VDR transfection into CV-1 cells, $1,25(OH)D_3$ at 10 nM induced the reporter expression by more than 150-fold compared with the induction by $1\alpha(OH)D_5$ at the same concentration. The results indicate that, at equimolar concentrations, $1,25(OH)_2D_3$ is more potent in transactivating the *VDRE* reporter gene than $1(OH)D_5$. This is consistent with the earlier findings that, in mouse mammary gland organ cultures, the D_5 analogue is required at a log molar higher concentration to achieve similar effects to those observed with $1,25(OH)_2D_3$. The advantage, however, is that the D_5 analogue does not induce unwarranted toxicity which is often associated with 1,25-dihydroxyvitamin D_3. These studies collectively indicate that the vitamin D_5 series of agents mediate their action via the same VDR-mediated mechanism that is operative with the active metabolite of vitamin D_3.

Acknowledgements

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Differentiation of human breast carcinoma cells by a novel vitamin D analog: 1α-hydroxyvitamin D_5

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Abstract. The active metabolite of vitamin D, 1α,25-dihydroxyvitamin D_3, can induce differentiation in breast cancer cells; however, it is hypercalcemic *in vivo*. Therefore, development of non-calcemic analogs of vitamin D has received considerable attention. Recently, we synthesized an analog of vitamin D [1α(OH)D_5] that exhibits much less calcemic activity than 1α,25-dihydroxyvitamin D_3. In this study, we evaluated the cell-differentiating action of 1α(OH)D_5 in breast cancer cells. Following 10 days treatment with 1α(OH)D_5 [(10^{-7} M) in UISO-BCA-4], we observed induction of intracytoplasmic casein, intracytoplasmic lipid droplets, ICAM-1, nm23, and specific biomarkers associated with breast cell differentiation. 1α(OH)D_5 treatment also showed induction of vitamin D receptor and TGFß1 proteins. UISO-BCA-4 cells pretreated for 10 days *in vitro* with 1 μM 1α(OH)D_5 failed to form tumors when transplanted into athymic mice. Similarly, 4 and 8 ng 1α(OH)D_5 treatment three times weekly inhibited the growth of UISO-BCA-4 cells injected into athymic mice. These results suggest that this new vitamin D analog may be of significant therapeutic value for breast cancer.

Introduction

In recent years, agents with antiproliferative and differentiating properties have been the primary focus of research in the areas of cancer therapy and prevention. Vitamin D has long been recognized as one of the most potent differentiating agents. Its potential role as a chemopreventive agent has also been proposed (1,2). Several epidemiological studies have shown a negative correlation between incidence of colon cancer and either serum vitamin D levels or intake of vitamin D (3-6). An active metabolite of vitamin D, 1α,25$(OH)_2$ vitamin D_3, has been reported to have antiproliferative and differentiating effects on various cell types (7-12).

One major factor limiting the successful use of vitamin D or its metabolites in cancer prevention or therapy is its calcemic activity. Treatment of experimental animals with vitamin D or its analogs results in significantly increased plasma calcium levels (13,14). The concentration needed to cause reduced growth of neoplastic cells would be sufficient to make the animal hypercalcemic and cause death. This unacceptable side effect has precluded use of vitamin D compounds in cancer prevention and therapy. One approach taken to circumvent this problem is to generate new synthetic chemicals that preserve vitamin D's growth suppressive/differentiating activity but reduce its calcemic activity.

In recent years, various synthetic analogs with no calcemic activity have shown potent growth-inhibitory and cell-differentiating effects *in vitro* and *in vivo* in experimental systems. For example, a hexafluoro derivative of vitamin D_3 developed by Hoffman La Roche (RO24-5531) has no calcemic activity and is reported to reduce mammary carcinogenesis (15). Similarly, analogs such as 22-oxa-calcitriol and EB1069 have shown chemopreventive effects with reduced calcemic activity (16,17). Yet very little is known about the effects of these agents on breast cancer cell differentiation and growth in athymic mice. In addition to vitamin D_2 and D_3, other series of vitamin D, vitamin D_4, D_5, and D_6 have been identified, but no synthetic analogs of these compounds have yet been synthesized or evaluated. Recently, we synthesized an analog of vitamin D from a vitamin D_5 series, 1α-hydroxyvitamin D_5, which is relatively less toxic than 1,25-dihydroxyvitamin D_3 and which exhibited potent chemopreventive effects in mammary gland organ cultures (18). In the present study, we examined the antiproliferative and cell-differentiating actions of a novel vitamin D analog on a human breast carcinoma cell line; we then compared these effects with those of 1,25$(OH)_2D_3$ and RO24-5531 in breast cancer cells.

Materials and methods

Human breast carcinoma cell lines. All established breast cancer cell lines (MCF-7, MDA-MB-231, MDA-MB-468, MDA-MB-435, T47D, and ZR-75-1) were obtained from the American Type Culture Association (ATCC), Rockville, MD.

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All cell lines were maintained in MEM-E containing 10% FBS. UISO-BCA-4 was established in our laboratory from metastatic pleural fluid obtained from a 56-year-old woman with a confirmed diagnosis of breast carcinoma. Patient's primary tumor was negative for estrogen and progesterone receptors, contained mutant p53, and was moderately positive for Her-2 protein. The cell line is originally established and continuously maintained in MEM-E [Minimum Essential Medium with Earle's salt, containing essential amino acids, glutamine, streptomycin, fungizone, and fetal bovine serum (10%)]. At present, the cell line is at passage 53 and is continuously passaged and maintained in culture. As a routine procedure, the cell line is screened periodically for mycoplasma contamination using a Micotrim Test Kit (Henna Media Inc., Berkeley, CA). The established cell line is confirmed to originate from human tissue by LDH isoenzyme analysis, whereas the breast origin was confirmed by HMFG immunostaining and electron microscopic details (19,20). No estrogen or progesterone receptor contents were detected in the cells as determined immunohistochemically using specific antiserum obtained from Dako Corp. (Carpinteria, CA). *In vivo*, UISO BCA-4 cells form palpable tumors when the cells are injected mixed with Matrigel. In all cell lines, ER, PR, P53 and VDR contents were examined by immunohistochemistry; in our laboratory, MCF-7 and ZR-75-1 were found ER^+PR^+, p53-negative (contained wild-type p53), and VDR^+. T-47D was ER^-PR^+, P53-positive (contained mutant p53), and VDR^+. All other cell lines (MDA-MB-231, MDA-MB-435, MDA-MB-468, and UISO-BCA-1) were ER^-PR^-, contained mutant p53, and showed low or undetectable VDR. UISO-BCA-4 was ER^-, PR^-, p53-positive, and positive for VDR protein.

In vitro effect of 1α(OH)D_5. 1,25(OH)D_3 and 1α(OH)D_5 were purchased from Steroids, Ltd. (Chicago, IL). RO24-5531 was obtained from NIH, Bethesda, MD. In order to determine the effects of 1α(OH)D_5 on cell growth, cells were plated (20,000/well) in 24-well tissue culture plates in MEM-E containing 5% DCC (Dextran-coated charcoal) treated with FBS alone or containing 1α(OH)D_5 ranging from $1x10^{-9}$ to $1x10^{-6}$ M. The cells were incubated at 37°C in an atmosphere of 5% CO_2 and 95% air; the medium was changed on days 4 and 7 after seeding. The cells in the control and treatment groups were trypsinized and counted using a coulter counter on day 10 after seeding. Results were calculated as percent of control. All compounds were initially dissolved in 100% ethanol and then added to the medium. The final ethanol concentration in the medium did not exceed 0.01%. For initial screening of growth-inhibitory action of 1α(OH)D_5 in various cell lines, cells were incubated for 10 days with 1 μM concentration of analog.

Immunohistochemical detection of various biomarkers. Immunohistochemical tests for various antigens in cells were performed by a modified indirect immunoperoxidase method with a streptavidin biotin complex kit (Dako Corp., Carpinteria, CA). Primary antibodies to specific antigens were obtained from the following suppliers: antihuman casein from Seralab (Sussex, England); ICAM-1, VDR, polyclonal antibodies for nm23 from Neomarkers (Fremont CA); and

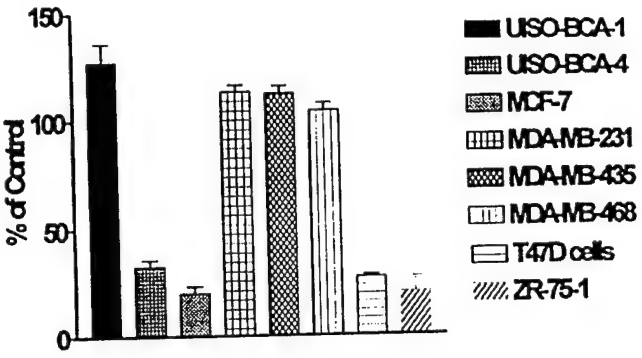

Figure 1. Effect of 1α(OH)D_5 on the growth of various breast cancer cell lines. Cells at 20,000/well density were incubated with 1 μM concentration of 1α(OH)D_5. Results are reported as perecentage ± SEM of vehicle treated control.

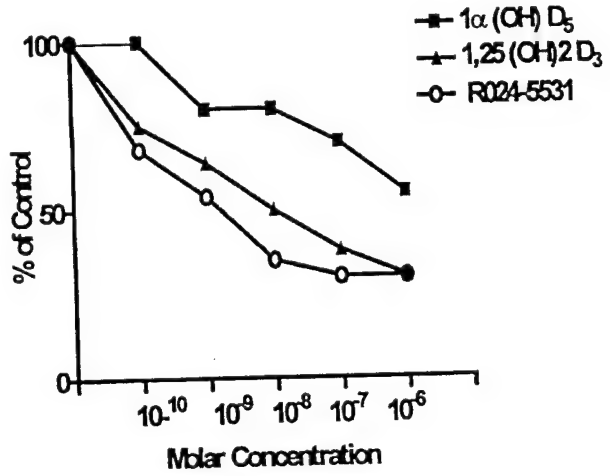

Figure 2. Dose-dependent effect of 1α(OH)D_5, 1,25$(OH)_2D_3$, and RO24-5531 on the growth of UISO-BCA-4 cells. The cells were incubated with different molar concentrations of 1α(OH)D_5. Results are represented as % of control.

TGFß1 from Promega Corporation (Madison, WI). The cells were first cultured on coverslips in the standard culture medium. After 24 h, cells were either treated with medium containing 1α(OH)D_5 or vehicle for 10 days at 37°C. At the end of the incubation, cells were washed with ice-cold phosphosaline buffer and fixed in buffered formalin (5 min), ice-cold methanol (3 min), and acetone (2 min). To block non-specific antibody binding, cells were treated with commercially available (Dako Corp. Carpinteria, CA) appropriate blocking reagent and then incubated with primary antibody or non-specific mouse/rat IgG (appropriate according to the primary antibody source, as controls). Following incubation, cells were washed with PBS, then incubated with biotinylated anti-mouse/rat link antibody and peroxidase-conjugated streptavidin. Immunoreactivity to primary antibody was visualized using 3-amino-9-ethyl-carbazole as chromogen.

Visualization of intracytoplasmic lipid droplets. Cells plated on Nunc coverslips were incubated either in the presence or absence of vitamin D analog. Following 10-day incubation, coverslips were rinsed twice with PBS, then fixed in ice-cold methanol. Following fixation, cells were placed in propylene glycol, then stained with oil-red O reagent (Rowley Biochemical Institute, Rowley, MA). The cells were rinsed with isopropyl alcohol, counterstained with hematoxylin, and mounted in aqueous mounting medium.

In vivo growth of UISO BCA-4 cells pretreated in vitro with 1α(OH)D_5. UISO-BCA-4 cells were pretreated with 1α(OH)D_5 (1 μM) for 10 days. Following the treatment, cells (1 million/animal) were mixed with growth factor-reduced Matrigel (Collaborative Biomedical Products, Becton-Dickinson Labware, Bedford, MA, 1:1 vol.). Cells were injected s.c. into female athymic mice. We used reduced growth factor-containing Matrigel to eliminate the possible influence of various growth factors on cells *in vivo*. Animals were monitored for the development of palpable tumor at the injection site. Any lesions or tumor tissues present were processed for histopathological evaluations.

Effect of 1α(OH)D_5 on the growth of UISO-BCA-4 cells in vivo. We determined the effect of vitamin D analog on the growth of UISO-BCA-4 cells in athymic mice. The animals were obtained from Frederick Cancer Research Facility, Bethesda, MD. UISO-BCA-4 breast carcinoma cells were suspended in an HBSS:matrigel (1:1 volume) mixture, then injected (1 million/animal) s.c. into the dorsal region of 3- to 4-week-old female Balb/c athymic mice. In order to obtain optimal tumor growth in the control group, we used regular Matrigel (Collaborative Biomedical Products, Becton-Dickinson Labware, Bedford, MA) matrix for this experiment. Animals were injected s.c. with 1α(OH)D_5 (4 and 8 ng/animal) three times weekly. All compounds were dissolved in ethanol, then diluted with normal saline solution. The control group received vehicle only. All animals were examined three times weekly for the development of palpable tumors at the site of injection or other sites. After six weeks of treatment, animals were sacrificed and tumor/lesion at the site of injection was excised and processed for histological examinations.

Results

Effect of 1α(OH)D_5 on in vitro cell growth and morphology. Initially, we examined the effect of 1α(OH)D_5 on various new and established cell lines. Cells were incubated with 1 μM 1α(OH)D_5 for 10 days. 1α(OH)D_5 had significant growth-inhibitory action on MCF-7, ZR-75-1, T47D, and UISO-BCA-4 cells (all VDR-positive). All other cell lines (negative/low VDR content) studied showed no significant growth inhibition following treatment with the analog (Fig. 1).

In order to avoid interference of ER, we further studied the effect of various concentrations of 1α(OH)D_5 on ER^-PR^- and VDR^+ UISO-BCA-4 cells. All three compounds tested showed dose-dependent growth inhibitory action; however, 1α(OH)D_5 had a weaker effect than 1,25$(OH)_2D_3$ and RO24-5531. At 1 μM concentration, 75%, 78%, and 45% inhibition of cell growth was noted with 1,25$(OH)_2D_3$, RO24-5531, and 1α(OH)D_5 respectively (Fig. 2). In addition to growth inhibition, the cells growing *in vitro* following 10 days exposure to 1 μM 1α(OH)D_5, 1,25$(OH)_2D_3$, and RO24-5531 induced typical morphological characteristic changes. For example, UISO-BCA-4 cells treated with 1α(OH)D_5, 1,25$(OH)_2D_3$, and RO24-5531 showed thin lacy cytoplasm and contained numerous intracytoplasmic vacuoles. The later changes were more pronounced in the cells treated with 1 μM 1α(OH)D_5. Occasionally, numerous fragmented nuclei were observed (Fig. 3). MCF-7 cells also showed similar morphological features following 1α(OH)D_5 treatment (data not shown).

Effect of 1α(OH)D_5 on various biomarkers associated with cell differentiation. We examined the effects of 1α(OH)D_5 on various biomarkers associated with breast cell differentiation in UISO-BCA-4 cells. All test compounds increased membrane-specific expression of ICAM-1 protein. Both RO24-5531 and 1α(OH)D_5 had significant effects on expression of ICAM-1 (Fig. 4). Similarly, an increase in intracytoplasmic casein granules was observed in cells treated with all vitamin D analogs (Fig. 5). We also determined the effect of vitamin D analogs on nm23 protein expression in UISO-BCA-4 cells. We observed intense immunoreactivity to nm23 antibody in cells incubated with 1,25$(OH)_2D_3$ and 1α(OH)D_5. The antibody used against nm23 in our study is known to cross-react with two different proteins: nm23-1 and nm23-2 (Fig. 6). To determine which of these two proteins is increased after 1α(OH)D_5 treatment, we performed Western blot analysis on cell lysates. Both nm23-1 (M.W. 22,000) and nm23-2 (M.W. 18,000) were higher in 1α(OH)D_5-treated cells than in untreated control cells (data not shown).

Staining of lipids by oil-red O reagent showed many large lipid droplets in 1α(OH)D_5-treated cells compared to the presence of occasional lipid droplets in control untreated cells (Fig. 7). Similarly, MCF-7 cells treated with 1α(OH)D_5 showed higher lipid accumulation than control cells (data not shown). We also examined the effect of all three test compounds on those cell lines (MDA-MB-231, UISO-BCA-1) which were found to be unresponsive in growth assay to vitamin D treatment. No significant changes in expression of ICAM-1, casein, nm23, or lipid content were observed in the latter cell lines (data not shown).

Effect of 1α(OH)D_5 on various markers associated with vitamin D action. Vitamin D is thought to exert its effect through its interaction with vitamin D receptors (VDR). We studied the effects of 1α(OH)D_5 on VDR status in vitamin D-treated UISO-BCA-4 cells. Following the treatment of UISO-BCA-4 cells with 1 μM 1α(OH)D_5/1,25$(OH)_2D_3$ for 10 days, intense nuclear staining for VDR was observed. Control untreated cells had no staining for VDR (Fig. 8A). Vitamin treatment also resulted in enhanced expression of TGFß1 protein in these cells (Fig. 8B). Further studies in all other cell lines showed that increased expression of VDR and TGFß1 was limited to those cells originally expressing immunohistochemically detectable VDR.

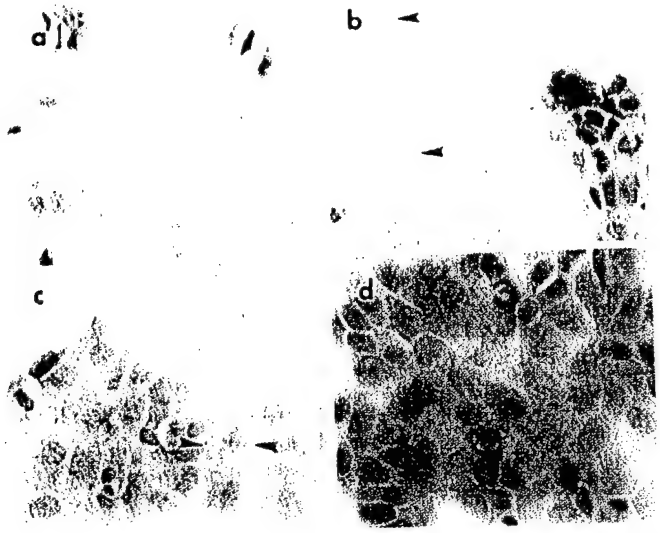

Figure 3. Morphology of UISO-BCA-4 cells following 10 days *in vitro* exposure to vehicle alone (a), 10^{-6} M 1α(OH)D_5 (b), 100 nM 1,25$(OH)_2D_3$ (c) and 1 μM RO24-5531 (d). Arrows show intracytoplasmic vacuoles.

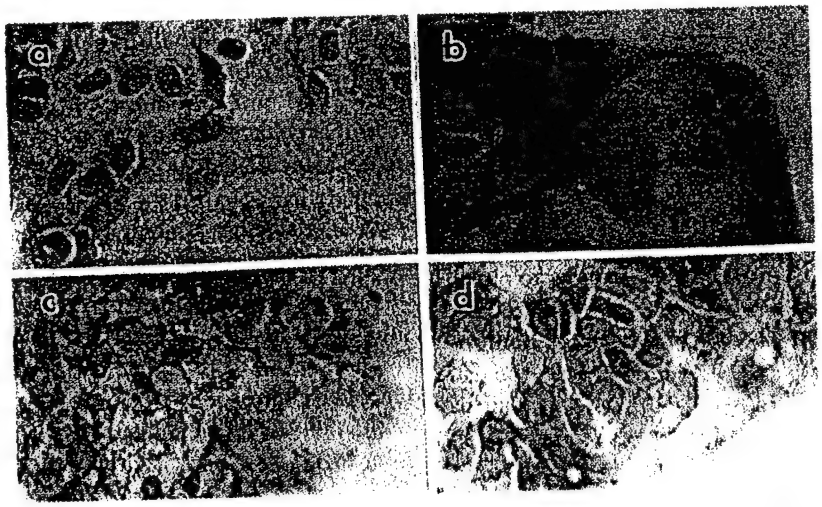

Figure 4. Immunohistochemical staining for ICAM in cells treated with vehicle (control) (a), 1α(OH)D_5 (b), 1,25$(OH)_2D_3$ (c), and RO24-5531 (d).

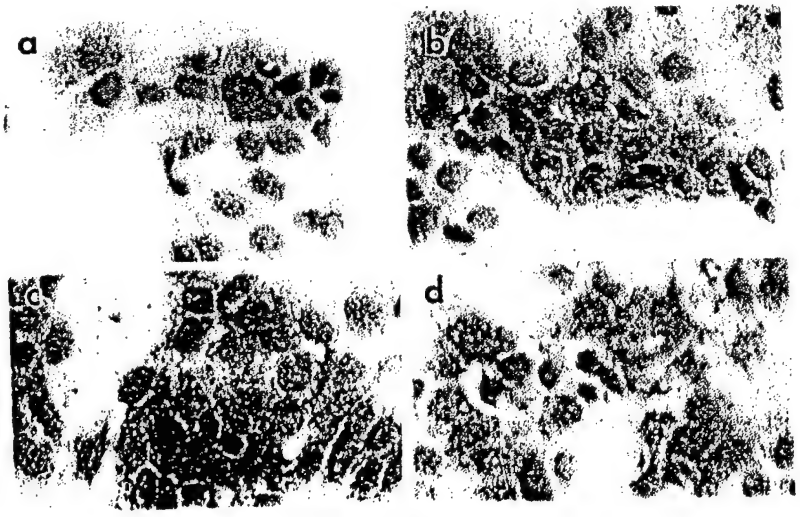

Figure 5. Immunohistochemical staining for casein in UISO-BCA-4 cells treated with vehicle (control) (a), 1α(OH)D_5 (b), 1,25$(OH)_2D_3$ (c), and RO24-5531 (d).

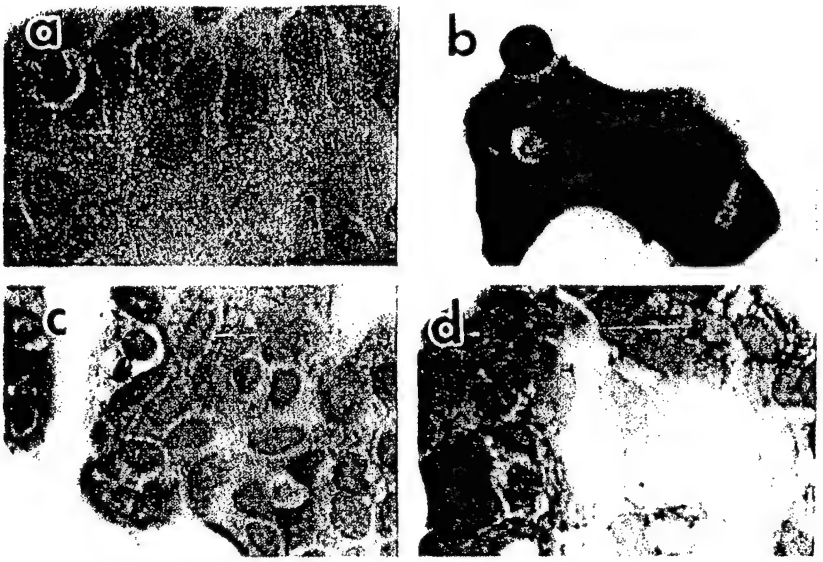

Figure 6. Immunohistochemical staining for nm23 protein in UISO-BCA-4 cells treated with vehicle (control) (a), $1\alpha(OH)D_5$ (b), $1,25(OH)_2D_3$ (c), and RO24-5531 (d).

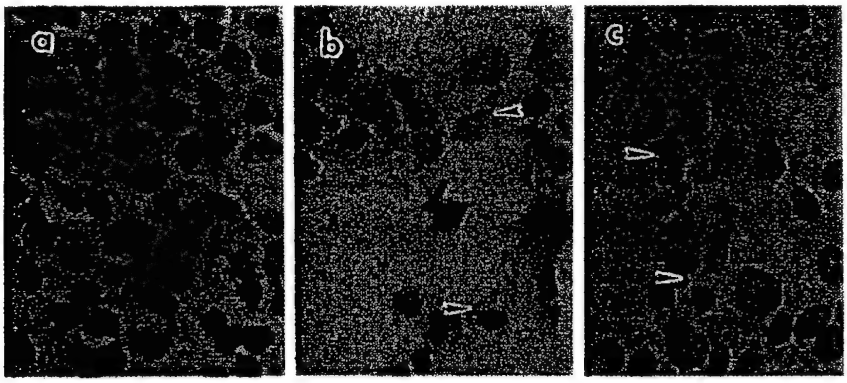

Figure 7. Staining for lipid contents by oil red O in cells treated with vehicle (a), $1\alpha(OH)D_5$ (b), and $1,25(OH)_2D_3$ (c). Arrows show lipid droplets.

Effect on in vitro/in vivo growth of UISO-BCA-4 cells pretreated with 1α(OH)D₅. To determine whether cells treated *in vitro* with $1\alpha(OH)D_5$ resume original growth ability after discontinuation of $1\alpha(OH)D_5$, we examined *in vitro* and *in vivo* growth of UISO-BCA-4 cells. UISO-BCA-4 cells were cultured for 10 days in only vehicle or in medium containing either $1\alpha(OH)D_5$ (1 μM) or $1,25(OH)_2D_3$ (10^{-7} M). The experiment was performed in 2 sets with 4 individual observations in each treatment group. The first set was terminated after 10 days. In the second set, all treatment groups received regular medium containing 15% FBS. The cells were allowed to grow for 10 more days, and the growth of cells was assessed in each group as a percentage of control of initial plating. As shown in Fig. 9, cells in the control vehicle group grew almost threefold after 10 days incubation in stripped serum medium. When these cells were transferred to regular medium (15% serum), 7-fold growth was noted within 10 days. On the other hand, cells receiving $1,25(OH)_2D_3$ increased to 1.5-fold which was further increased to two-fold in regular medium. We transplanted treated and untreated cells in 3- to 4-week-old female athymic mice. The cells were mixed with Matrigel with reduced growth factor in order to minimize the effects of growth factors present in the Matrigel. In the control group, small palpable tumors grew in 5/5 animals; in one of five animals receiving $1\alpha(OH)D_5$-treated cells, only a flat, scab-like structure was observed. We excised the tumor/structure formed at the site of inoculation and performed histological evaluation. In animals receiving untreated cells, tumors showed numerous clusters of cells embedded in the stroma. The cells had large distinct nuclei and abundant cytoplasm. On the other hand, animals receiving $1\alpha(OH)D_5$ had only a few isolated patches of cells, with small nuclei and no clear appearance of cytoplasm (Fig. 10).

Effect of 1α(OH)D₅ on in vivo growth of UISO-BCA-4 cells. To determine the effect of $1\alpha(OH)D_5$ on *in vivo* growth, UISO-BCA-4 cells were injected s.c. into 3- to 4-week-old female

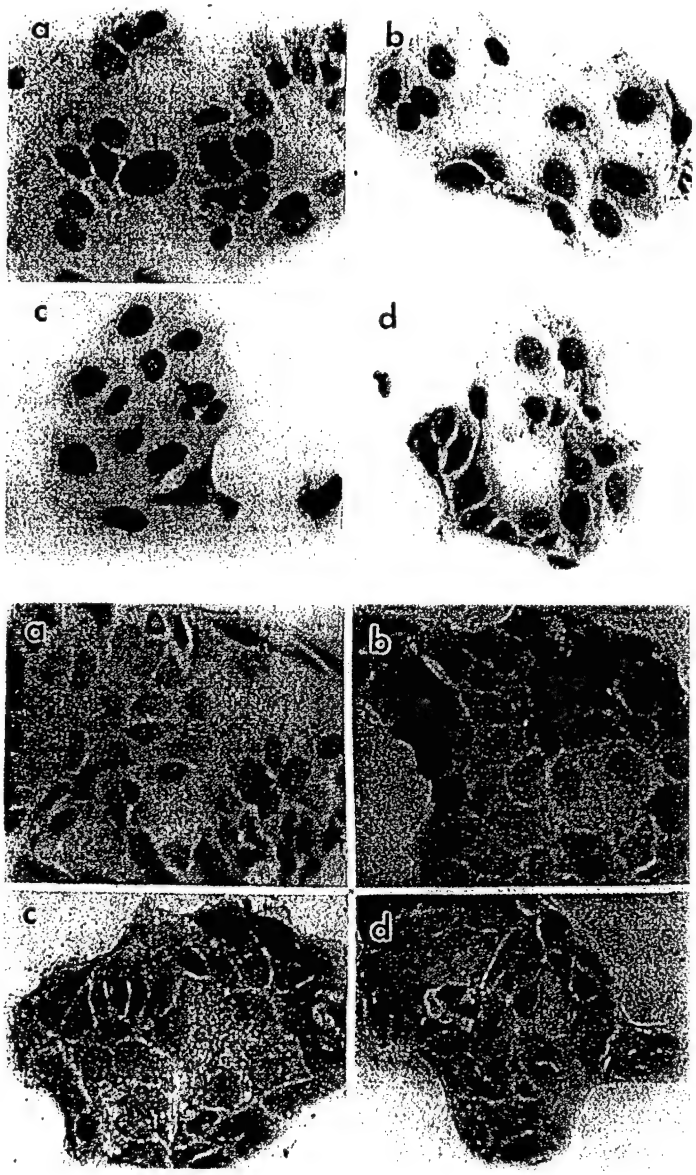

Figure 8. A, Induction of nuclear VDR in UISO-BCA-4 cells following treatment with vitamin D analog. a, vehicle treated control; b, 1α(OH)D_5; c, 1,25$(OH)_2D_3$; d, RO24-5531. B, Induction of nuclear TGFß1 protein in UISO-BCA-4 cells following treatment with vitamin D analog. a, vehicle treated control; b, 1α(OH)D_5; c, 1,25$(OH)_2D_3$; d, RO24-5531.

athymic mice. In order to obtain optimal tumor growth, cells were mixed with regular Matrigel containing growth factors. Animals received 1α(OH)D_5 (at 4-8-ng/animal dose) or vehicle alone s.c. three times weekly. In the control group, tumor growth at the site of injection was observed in 5/5 animals. Small but distinct palpable tumor developed after two weeks, and it continued to grow at a slower rate. Histological studies of this tumor indicated that the tumor was highly cellular (similar to the original patient's tumor) and contained clusters of breast cancer cells embedded in the host stroma (Fig. 11). In 2/5 control animals, metastasis to the lymph nodes were observed. The tumors in lymph nodes were larger and palpable. On the other hand, a scab-like structure was observed at the inoculation site only in 1/5 animals in both 4 and 8 ng (one in each dose) 1α(OH)D_5-treated animals. None of these 1α(OH)D_5-treated animals showed metastatic tumors in the lymph nodes. Histologically, the scab-like tumor observed in 1α(OH)D_5-treated animals showed few human breast carcinoma cells embedded in the stroma (Fig. 11).

Discussion

An active metabolite of vitamin D_3, 1α,25-dihydroxyvitamin D_3, regulates calcium homeostasis and bone metabolism. In addition, several reports have shown that 1α,25-dihydroxyvitamin D_3 or its analogs are involved in regulation of cell proliferation and differentiation (7-9,12,21). The effects of vitamin D and its analogs have been studied largely using the structural modifications of vitamin D_3. Vitamin D is structurally classified into D_2 through D_6 series. Vitamin D_3 is the most widely used series of compounds since 1α,25$(OH)_2D_3$

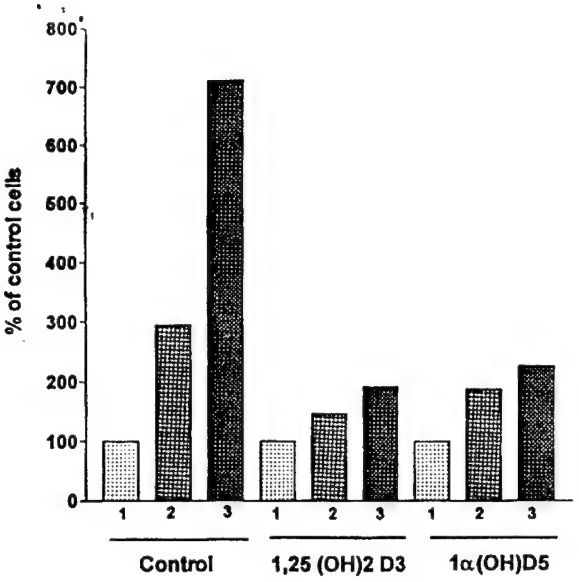

Figure 9. *In vitro* growth of UISO-BCA-4 cells pretreated with charcoal-stripped serum containing medium alone or with $1,25(OH)_2D_3$ (10^{-3} M) or $1\alpha(OH)D_5$. Column 1 represents initial plating (cells at the beginning of the experiment). Column 2 represents the percentage of cells (compared to initial plating) after indicated treatment. Column 3 represents the percentage of cells following 10 days treatment received as in Column 2, but then incubated an additional 10 days in regular serum containing medium.

is considered an active metabolite of vitamin D. Therefore, analogs have been synthesized to reduce the toxicity of vitamin D. We have currently focused our attention on designing molecules that are analogs of the D_5 series of compounds, $1\alpha(OH)D_5$ is one such analog. We recently showed that this analog is less calcemic than dihydroxy D_3 and inhibits development of carcinogen-induced mammary lesions in mouse mammary gland organ cultures (18). It also induced vitamin D receptors and TGFß1 at a non-toxic concentration. These results suggest that 1α-hydroxyvitamin D_5 may serve as an excellent chemopreventive agent.

Although the effects of vitamin D on growth inhibition and chemoprevention have been studied, its ability to differentiate breast cancer cells has not been studied critically. Most of the commercially available human breast carcinoma cell lines reported to be responsive to vitamin D metabolite, $1,25(OH)_2D_3$, or other vitamin D analogs (8,22) are ER^+PR^+. The presence of steroid receptors in breast cancer makes it difficult to study the cell-differentiating action of these vitamin D-related compounds, because $1,25(OH)_2D_3$ (a vitamin D metabolite) is known to interact with estrogen response element (ERE) at the molecular level and to directly influence ER levels in ER^+ cells (23,24). Additionally, p53 status (wild-type) also influences the effect on the cell growth

Figure 10. Histology of lesions formed in athymic mice following transplantation of control untreated cells (C) and UISO-BCA-4 cells treated for 10 days with 10^{-6} M $1\alpha(OH)D_5$ (D_5) cells. UISO-BCA-4 cells were incubated in the basal culture medium or medium supplemented with 10^{-6} M $1\alpha(OH)D_5$ for 10 days. Following incubation, cells were suspended in HBSS and mixed with 1:1 volume of Matrigel. The cells were injected s.c. into the dorsal flank region of 4- to 6-week-old female athymic mice. After 60 days, lesions that developed at the site of injection were processed for histological studies.

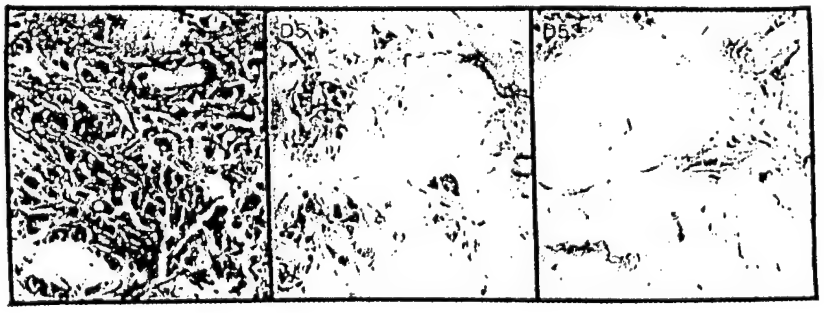

Figure 11. Effect of *in vivo* administration of $1\alpha(OH)D_5$ on UISO-BCA-4 cells transplanted into athymic mice. UISO-BCA-4 cells (1 million/animal) were injected s.c. into the dorsal flank region of female athymic mice. Animals received, by s.c. injection, vehicle (C) or 4 ng D_5 (center) or 8 ng D_5 (right) of $1\alpha(OH)D_5$ three times per week. After 60 days of treatment, lesions that formed at the site of injection were processed for histological studies.

by these compounds. In this study, we investigated the effects of $1\alpha(OH)D_5$ (our newly synthesized vitamin D analog), $1,25(OH)_2D_3$ (a vitamin D metabolite), and RO24-5531 (a vitamin D analog) on the differentiation of UISO-BCA-4 cells. Initially, we evaluated the effect of $1\alpha(OH)D_5$ on the growth of various breast carcinoma cell lines. The growth-inhibitory action of $1\alpha(OH)D_5$ was observed in all four VDR^+ cell lines. To study the cell-differentiating action, we selected UISO-BCA-4 cells. These cells are ER^- and PR-negative, yet VDR-positive. These characteristics allowed us to evaluate the effect of this analog without its interaction with ER or ERE. Since the cell line contains mutant p53 protein, we could determine the p53-independent effects of vitamin D on these cells. Following 10 days exposure to all vitamin D-related compounds, we observed enhanced expression of various biomarkers (such as nm23, ICAM-1, intracellular casein, and lipid content) generally associated with breast cell differentiation.

Since the induction of differentiation was achieved by $1\alpha(OH)D_5$, a question arises whether differentiated breast cancer cells have lost tumorigenic potential and therefore are less likely than the parent cells to grow into breast tumors. We examined this possibility by treating BCA-4 cells with $1\alpha(OH)D_5$ at a concentration that induces differentiation after 10 days incubation in culture. When injected in athymic mice, vitamin D_5-treated cells failed to form tumors. These results suggest not only that $1\alpha(OH)D_5$ is a potent inducer of cell differentiation, but also that it can provide chemo-therapeutic effects against breast cancer growth.

Previously, 1α,25 dihydroxyvitamin D_3 and its various synthetic analogs have been shown to have growth-inhibitory and cell-differentiating actions *in vitro* in human breast carcinoma cell lines (7-10,12). We studied the effects of $1\alpha(OH)D_5$ on a human breast carcinoma cell line transplanted in athymic mice. Animals were injected with either 4 or 8 ng (25-50 times lower than optimal tolerated dose determined in screening assay) of vitamin D_5 three times weekly for 60 days. Results showed that $1\alpha(OH)D_5$ inhibited the growth and metastasis of human breast cells transplanted in mice. Recently, we evaluated the toxicity of this agent in athymic mice (unpublished data). Animals were injected three times per week with 10, 25, 50, 100, and 200 ng of vitamin D_5. At the highest concentration (200 ng), some toxicity was evident in terms of less body weight gain than in control mice. However, the agent was non-toxic at 100 ng concentration (data not shown).

Although the effects of vitamin D_3 metabolite and its various analogs have been studied in various cell types, its mechanism of action is still not fully understood. Vitamin D_3 analogs bind to specific nuclear receptor VDR (25). Receptor ligand complexes alter the transcription of a variety of genes through binding to specific regulatory elements in the target promoter sequences (2). VDR is induced in many cell types following VDR-ligand interaction. We observed induction of VDR in UISO-BCA-4 cells after $1\alpha(OH)D_5$ treatment. $1\alpha(OH)D_5$ also induced expression of TGFß1 protein in UISO-BCA-4 cells. TGFß1 has been shown to have anti-proliferative and cell-differentiating actions in many malignant cell types (26-29). Our results in UISO-BCA-4 cells suggest that the effects of vitamin D on cell differentiation may be mediated by vitamin D-induced VDR and subsequent induction of negative growth regulatory factor TGFß1 in ER^- cells.

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Effect of combination treatment with Her-2 antibody and 1α(OH)D_5 in human breast carcinoma

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Summary

1,25$(OH)_2$ vitamin D_3, an active metabolite of vitamin D, shows antiproliferative and cell-differentiating actions in human breast carcinoma cells; however, its hypercalcemic property has prevented its use as a therapeutic or chemopreventive agent. Recently, we identified a vitamin D analog, 1α(OH)D_5, that induced cell differentiation and had potent antiproliferative actions in MCF-7, T-47D, and UISO-BCA-4 breast cancer cells, but showed no calcemic activity in mice and rats. In the present study, we further evaluated the cell-differentiating and therapeutic effects of this compound alone and in combination with Her-2 antibody in two human breast carcinoma cell lines (ZR-75-1, BT-474) expressing high to moderate levels of Her-2 receptors. *In vitro*, 1α(OH)D_5 showed growth inhibitory effect at 0.1 μM concentration. Incubation of cells in the presence of Her-2 antibody (1.5 μg/ml) had no significant effect on the growth; however, a combination of both 1α(OH)D_5 and Her-2 antibody significantly reduced the growth of BT-474 cells compared to that observed following treatment with either agent alone. 1α(OH)D_5 or Her-2 antibody alone for 7 days induced cell differentiation, as evident from accumulation of intracellular lipid droplets. Treatment of cells with both 1α(OH)D_5 and Her-2 antibody showed further increase in lipid droplet accumulation. *In vivo*, 1α(OH)D_5 supplemented in the diet reduced the growth of cells transplanted into female athymic mice. Combination treatment with Her-2 antibody (given i.p.) and dietary supplementation of 1α(OH)D_5 was more effective than either agent alone in reducing the tumor growth, and the latter effect was irreversible, as tumors failed to show continued growth after discontinuation of treatments.

Introduction

Recently, identification of naturally occurring or synthetic agents with both antiproliferative and differentiating properties has been a focus of research in the areas of cancer therapy and prevention. Vitamins A and D are recognized as the most potent cell-differentiating and growth-inhibiting agents for breast cancer cells.[1,2,3,4,5,6,7,8,9,10,11,12,13]

1,25 (OH)2 vitamin D_3, an active metabolite of vitamin D, has been reported to have antiproliferative action in various malignant cells.[6,7,9,13] However, use of vitamin D metabolite as a therapeutic agent is limited due to its calcemic property.[14,15,16,17] Recently, we have shown that 1α(OH)D_5, a synthetic analog of 1α(OH)D_5,[12,13] has potent antiproliferative and cell-differentiating actions in human breast cancer cells.[12,13] 1α(OH)D_5 induced expression of various proteins associated with breast cancer cell differentiation (nm23, ICAM-1, nm23, alpha2 integrin, intracytoplasmic protein) and also showed increased accumulation of lipid droplets in MCF-7, UISO-BCA-4, and T-47D cells.[12,13] *In vitro* experiments in various cell lines suggested that the effect of 1α(OH)D_5 is mediated through VDR.[12] *In vivo*, 1α(OH)D_5 administered s.c. reduced or inhibited growth of human breast carcinoma cells transplanted into athymic mice.[12] 1α(OH)D_5 also has potent chemopreventive action in experimental animal models.[18] *In vitro* in the mouse mammary gland organ culture system, 1α(OH)D_5 was effective at inhibiting the development of carcinogen-induced mammary gland lesions.[18] In rats, 1α(OH)D_5 was highly effective at reducing DMBA-induced mammary tumors.[19]

Even though, in the last decade, considerable progress has been made in treatment, diagnosis, and management of breast cancer, 30-40% of patients fail to respond to current chemotherapeutic modalities.[20] Thus, a major challenge for oncologists is to identify novel, effective therapies for highly aggressive breast cancers.[20] Overexpression and alteration of

oncogenes encoding growth factors and growth factor receptors have been shown to play an important role in the pathogenesis and progression of various cancers, including breast cancer.[21] Among the various oncogenes identified in breast cancer, the Her-2 gene has received most attention.[22,23,24,25] The Her-2 gene encodes a 185 KDa transmembrane glycoprotein receptor.[22] In 20% to 30% of human breast carcinomas, overexpression of Her-2 protein is observed.[25] Generally, patients with Her-2+ tumors have poor prognosis.[23,24,25] In experimental models, antibodies generated against the extracellular domain of Her-2 protein has shown potent therapeutic potential.[26,27,28,29,30,31,32,33] Combination treatment with Her-2 antibody and classical chemotherapeutic agents was more effective at inhibiting the growth of breast tumor xenograft than either agent given alone.[26,27,28,29,30,31,32,33] A recent phase II/III clinical trial with Trastuzumab/Herceptin (humanized Her-2 antibody) in patients with metastatic breast cancer indicated that Herceptin could be well tolerated and clinically effective in patients with Her-2 overexpressing metastatic breast cancer.[34,35,36,37,38,39] Combination treatment with a classical chemotherapeutic agent (Cisplatin) and Herceptin enhances the chemosensitivity better than the cisplatin given alone in patients with metastatic breast cancer and overexpressing Her-2 protein.[34] Even though Herceptin treatment has shown promising results, cardiac toxicity associated with Herceptin therapy has been a major concern for many oncologists.[40] Thus, there is a need to identify a highly effective nontoxic therapeutic modality.

In the present study, we have evaluated the therapeutic potential of 1α(OH)D_5 alone and in combination with low dose of Her-2 antibody in human breast carcinoma cells with Her-2 overexpression.

Materials and Methods

Human breast carcinoma cell lines: Breast carcinoma cell lines (BT-474, ZR-75- 1) were obtained from the American Type Culture Association (ATCC), Rockville, MD. All cell lines were maintained in MEM-E (Minimum Essential Medium containing Earle's salt) supplemented with 10% Fetal bovine serum (FBS), essential amino acids, glutamine, and streptavidin fungizone.

Vitamin D analog and Her-2 antibody: 1α(OH)D_5 was purchased from Steroids Ltd. (Chicago, IL). For both *in vitro* and *in vivo* studies, 1α(OH)D_5 was originally dissolved in 100% ethanol. For *in vitro* studies, stock solution was diluted and then added to the culture medium, so that the final concentration of ethanol in the medium was less than 0.1%. Antibody (BSA-free, azide-free) against the extracellular domain of Her-2 receptor used for both *in vivo* and *in vitro* studies was obtained from Neomarkers (Fremont, CA). Her-2 antibody (clone 9G6.10, ab-2) is reported to immunoprecipitate 160 KDa protein from extracts of Her-2-positive cells.[41]

The effect of 1α(OH)D_5 and Her-2 antibody on *in vitro* growth of BT-474 cells: For *in vitro* effect on growth, human breast carcinoma cells were plated at 10,000/well density in 24-well tissue culture plates. The cells were allowed to attach to the culture wells overnight in MEM-E containing 10% FBS. The following day, the medium was replaced with either control medium (MEM-E containing 5% Charcoal stripped FBS) or medium containing different concentrations of 1α(OH)D_5, Her-2 antibody, or Her-2 antibody + 1α(OH)D_5. Culture plates were incubated at 37°C in the atmosphere of 95% CO_2 and 5% O_2. Medium was changed with respective ingredients on day 4 following initiation of the treatment. The number of cells in each treatment group was counted on day 7 using a coulter counter. Data represent % of control and are calculated as mean $\pm$ SE of quadruple observation in each treatment group.

Morphological changes following 1α(OH)D_5, Her-2 antibody alone, or Her-2 antibody + 1α(OH)D_5 treatment: Cells growing in MEM-E (with 10% serum) were trypsinized and then plated on Nunc coverslip. After 24-48 hours, cells were cultured either in control medium or medium containing vitamin D analog. Medium was changed in each treatment group on day 4 of treatment initiation. On day 7, cells were washed with PBS, fixed in 10% buffered formalin and then dehydrated through the graded series of alcohol, and stained with Hematoxylin and Eosin by standard histological procedure.

The effect of 1α(OH)D5, Her-2 antibody, and combination of 1α(OH)D5 + Her-2 antibody on *in vivo* growth breast cancer cells: The effects of 1α(OH)D5, Her-2, and 1α(OH)D5 + Her-2 antibody were determined on BT-474 and ZR-75-1 cells transplanted into athymic mice. The animals were obtained from Frederick Cancer Research Facility, Bethesda, MD. BT-474 cells (1 million/animal) were suspended in a mixture of HBSS: Matrigel (1:1 vol/vol) then injected s.c. into the dorsal flank region of 3- to 4-week-old female Balb/c athymic mice. All animals received s.c. estrogen pellet (0.72 mg/animal, 60 days release, Innovative Research, Saratoga, FL). Animals were divided into various groups: 1) receiving regular powdered diet mixed with ethanol as vehicle; 2) receiving 1α(OH)D_5 (12.5 μg/kg diet)-supplemented powdered diet; 3) receiving i.p. injection of Her-2 antibody (5 μg/animal, once weekly) and regular powdered mouse diet as in group 1; 4) receiving Her-2 antibody (5 μg in 0.1 ml saline/animal, once weekly) i.p. as in group 3 but receiving diet supplemented with 1α(OH)D5. Groups 1 and 2 also received i.p. injection of saline (0.1 ml, once weekly).

Both control and 1α(OH)D5-supplemented diet were given to the animals in sterile food cups; an equal amount of food was placed in each cup. Food cups were protected from direct light exposure. Food cups were changed twice weekly. At the time of food cup change, diet

consumption in each group was estimated roughly based on unused food contents in the cups. All animals received water ad libitum. Each group consisted of a minimum of 5 animals. Animals were weighed and examined once weekly for growth of tumor at the site of injection. Once a palpable tumor developed, tumor size was monitored using calipers. Tumor volume was calculated as cm^3 using the formula, tumor volume = 3.14/6 x length x width x depth. Data represent mean + SE tumor volume (cm^3) in each group. Animals were sacrificed at the indicated time unless they appeared to be moribund or tumors showed sign of necrosis. At the termination, tumors were removed, fixed in 10% buffered formalin, and processed for histopathological and immunocytochemical studies.

Preparation of diet: For preparation of 1α(OH)D_5-supplemented diet, a known amount of 1α(OH)D_5 was dissolved in absolute ethanol and then mixed with powdered mouse chow (Teklad, Madison, WI) using diet mixer. Diet was stored in foiled containers to protect from light and stored at 4°C. Stability of 1α(OH)D_5 was determined periodically. An aliquot of the diet was extracted with methanol, and the extract was subjected to HPLC analysis. Control diet was mixed with ethanol (equal to that used for 1α(OH)D_5 diet) only. Ethanol from the diet mixtures was evaporated by placing it at room temperature for 20 min. in sterile culture hoods.

Lipid staining in BT-474 and ZR-75–1 cells: The cells were plated on Nunc coverslips and were allowed to attach to the coverslip for 48 hours. After 48 hours, cells were incubated at 37°C in MEM-E containing 5% charcoal-treated FBS alone or in the latter medium containing 1α(OH)D_5 (10^{-7}M), 1α(OH)D_3 (10^{-7}M), Her-2 antibody (1.5 μg/ml), or Her-2 antibody (1.5 μg/ml) + 1α(OH)D_5 (10^{-7}M) for 7 days. The medium was changed on day 4 after initiating treatment. At the end of incubation, cells were washed with PBS and fixed in 10% buffered formalin (5 min) and propylene glycol (3 min), then stained in oil-red O reagent (Rawley

Biochemical Institute, Rawley, MA). The cells were rinsed in isopropyl alcohol, counterstained in hematoxylin, and mounted in aqueous mounting medium.

Determination of serum calcium levels: Quantitative determination of serum calcium levels was performed by calorimetric analysis using calcium (Arsenazo III) reagent (Sigma Diagnostics, St. Louis, MO). Calcium in the serum reacts to the Arsenazo III reagent and forms purple-colored calcium-Arsenazo III complex. The intensity of the color is read at 600 nm and is evaluated in relation to the color intensity obtained by processing simultaneously known calcium standards (Sigma Diagnostics, St. Louis, MO).

Statistical Analysis: Differences in values obtained in each group were analyzed by the student's t-test. P value <0.05 was considered as statistically significant difference.

Results

The effect of 1α(OH)D_5, Her-2, or combination treatment on *in vitro* growth of breast cancer cells: In order to determine the effect of 1α(OH)D_5, Her-2 antibody, or Her-2 antibody +1α(OH)D_5, BT-474 cells were exposed for 7 days to control medium containing charcoal-stripped medium only or medium containing the test compounds. 1α(OH)D_5 treatment showed significant growth inhibitory action at high concentrations (10^{-7}M, 10^{-6}M). At low concentrations (10^{-9}M, 10^{-8}M), 1α(OH)D_5 showed no significant effect on cell growth. Similarly, 1,25 (OH)2 D_3 at 10^{-7}M concentration had a significant growth-inhibitory effect on BT-474 cells. BT-474 cells incubated with Her-2 antibody alone showed no significant effect on cell growth. When cells were exposed simultaneously to 1α(OH)D_5 + Her-2 antibody, we observed significant ($p<0.05$) reduction in cell number compared to that in the control group. Addition of Her-2 to 1α(OH)D_5 generated significantly better growth inhibitory effect than 1α(OH)D_5 treatment alone (Figure 1).

Morphological changes in BT-474 cells following treatment with 1α(OH)D_5, Her-2 antibody: BT-474 cells incubated in SS medium for 7 days showed large nuclei with the presence of 2-3 nucleoli. The occasional presence of small cytoplasmic vacuoles was observed. Following 7 days exposure to 1,25 (OH)2 D_3 or 1α(OH)D_5, BT-474 cells showed large nuclei, with 2-3 darkly stained nucleoli. In the cytoplasm, the presence of numerous vacuoles was observed. In cells treated with Her-2 antibody, no significant morphological changes were observed compared to control treatment (Figure 2).

The effect of 1α(OH)D_5, Her-2, and 1α(OH)D_5 + Her-2 antibody on the accumulation of lipid droplets: BT-474 cells were evaluated for the accumulation of lipid droplets following 7 days *in vitro* exposure to 1α(OH)D_5 and Her-2 antibody. As shown in Fig. 2, following

treatment with 1α(OH)D_5 (10^{-7}M), we observed increased accumulation of lipid droplets in the cytoplasm compared to cells incubated in the control medium. Incubation of cells in the presence of Her-2 antibody also showed increased lipid droplets compared to control. Addition of Her-2 antibody to 1α(OH)D_5 treatment showed further increase in lipid accumulation in these cells (Figure 3).

The effect of test compounds on *in vivo* growth of BT-474 cells transplanted into athymic mice: We used the nude mouse model to evaluate the effect of 1α(OH)D_5 on *in vivo* growth of BT-474 cells and ZR-75-1 cells. These two cell lines were selected based on expression of Her-2 and their ability to form tumors in the athymic mouse model. All animals received estradiol pellets to enhance the growth of tumor. Growth curves for mean tumor volume in each group are shown in Fig. 4. Growth of BT-474 cells was slow initially in both control and experimental groups. In the group receiving control diet, BT-474 tumors grew at a very slow rate initially for the first 20 days. After 20 days, the tumor attained exponential growth phase; by day 56, the mean tumor volume was 0.2 cm^3. In animals receiving 1α(OH)D_5, tumors grew at a lower rate during the first 15 days and achieved maximum tumor volume of approximately 0.03 cm^3 and then started to decline. At day 56 in animals whose diet was supplemented with 1α(OH)D_5, tumor volume was <0.02 cm^3, 10-fold smaller than that in the control group. Similarly, in animals receiving i.p. Her-2 antibody administration, tumors grew larger between days 20 and 56; at day 56, mean tumor volume in this group was 0.11 cm^3. Combined treatment with Her-2 antibody and dietary supplement of 1α(OH)D_5 showed further reduction in tumor volume compared to those in either 1α(OH)D_5 or Her-2 treatment alone (Figure 4).

We further examined the histopathology of tumors obtained at the time of termination. As shown in Figure 5, xenografts originated in animals receiving control diet showed highly

cellular morphology. In contrast, xenografts originated in animals treated with 1α(OH)D_5 showed few cell patches embedded in the Matrigel stroma. Xenografts originated in animals treated with i.p. administration of Her-2 antibody were histopathologically similar to those observed in animals receiving control diet without Her-2 antibody treatment. Interestingly, xenograft originated in animals receiving both 1α(OH)D_5-supplemented diet and Her-2 antibody administration showed few cells embedded in the matrix. Cells showed the morphology of differentiated cells.

We evaluated the effects of 1α(OH)D_5 and Her-2 antibody on ZR-75-1 cells transplanted in athymic mice. As shown in Figure 6, in the control group receiving regular powdered diet, tumors showed exponential growth from day 10-42. In animals receiving c-neu antibody but normal diet, tumor growth was slower than in the control group receiving no Her-2 antibody and regular diet. The group receiving diet supplemented with 1α(OH)D_5 had significantly ($p<0.05$) smaller tumor volume than that receiving control diet without 1α(OH)D_5 supplement. Similarly, mean tumor volume was significantly ($p<0.05$) smaller in the group of animals receiving combined 1α(OH)D_5 and Her-2 antibody treatment as compared to that observed in the control group, the group receiving Her-2 antibody alone, or the group receiving 1α(OH)D_5 dietary supplement alone (Figure 6). Histopathological details of the tumors obtained from the animals on 1α(OH)D_5 diet and those receiving the control diet are shown in Figure 7. Tumor from the control group consisted of tightly packed large epithelial cells with minimum inter cellular stromal component. Tumor cells had large prominent nucleus. In contrast, tumors from the 1α(OH)D_5-treated group showed the presence of small clusters of cells embedded in the stroma (mostly Matrigel). Cells were smaller in size, and nuclei were smaller than those on control

group. The occasional presence of cell clusters with morphology similar to that observed in control tumor was also observed (Figure 7).

In order to determine whether the growth inhibitory effect of 1α(OH)D_5 is transient, we switched all experimental groups (animals previously receiving Her-2 antibody alone, 1α(OH)D_5-supplemented diet, or Her-2 antibody + 1α(OH)D_5-supplemented diet) to control regular powdered diet on day 45. None of the groups received Her-2 antibody after day 45. Tumor volume was monitored periodically and recorded. As shown in Figure 8, after switching the animals to regular diet, all groups except the group receiving 1α(OH)D_5 + Her-2 antibody combination treatment showed accelerated tumor growth. At day 64, when the experiment was terminated, mean tumor volume in animals initially receiving combination treatment was significantly ($p<0.05$) smaller than in the three other groups (Figure 9). These results were further supported by histological examination of tumors in different groups. As shown in Figure 10, the histopathology of tumors in control, 1α(OH)D_5, or Her-2 antibody were similar, showing highly cellular histopathology. Tumors in animals who received combination treatment showed only a few cells embedded in stromal tissues.

Serum calcium levels in animals receiving different treatments: We determined serum calcium levels in animals receiving the different treatment regimens mentioned above. In animals bearing BT-474 tumors, mean serum calcium levels at the time of experiment termination were not significantly different in control vs. the group receiving 1α(OH)D_5 diet or only Her-2 antibody. In animals receiving combination therapy (Her-2 antibody + 1α(OH)D_5), serum calcium levels were higher ($p<0.05$) than those in the control group. Similar serum calcium profiles were observed in animals bearing ZR-75-1 xenograft (Figure 10). In general, no physically apparent symptoms of hypercalcemia were reported in any treatment group. Animals

appear to be active, and no signs of lethargy or dehydration were noticed, although mean body weight gain in groups receiving 1α(OH)D_5-supplemented diet was significantly ($p<0.05$) less than in those groups receiving control diet with no 1α(OH)D_5 supplement (Figure 11). One week after treatments ceased, body weights did not significantly differ between groups receiving control and 1α(OH)D_5-supplemented diet. Serum calcium levels were determined in groups of animals after discontinuation of the treatment, at the time of termination of the experiment. No significant difference in the serum calcium levels was observed in various treatment groups (Figure 12).

Discussion

1α(OH)D_5 is a synthetic analog that showed potent antiproliferative and cell-differentiating action in various human breast carcinoma cell lines. In the present study, we evaluated the therapeutic efficacy of 1α(OH)D_5 in human breast cancer cells showing overexpression of Her-2/neu protein. In breast cancer patients, overexpression of Her-2 protein in the tumor is reported to be associated with highly aggressive tumor type.[22,22,24,25] In general, patients with Her-2-overexpressing breast tumor fail to respond to the standard chemotherapeutic regimen.[22,24,25] Thus, the current research focus in clinical management of breast cancer is to design new highly effective therapies for such aggressive tumors, especially those overexpressing Her-2 receptor. Herceptin, a humanized antibody to the extracellular domain of Her-2 protein, has shown promising results in breast cancer patients.[34,36,37,38,39] However, phase III clinical trials recently completed show that antibody therapy in some women causes cardiac toxicity.[40] Thus, even though the therapeutic potential of herceptin is promising, it is desirable to identify new therapeutic strategies that will reduce the toxic side effects of antibody therapy without compromising its efficacy.

We studied the effects of 1α(OH)D_5 or Her-2 antibody alone or in combination in two well established human breast carcinoma cells lines: BT-474 and ZR-75-1. BT-474 cells show overexpression of Her-2 receptors, and ZR-75-1 cells have moderate expression of Her-2 receptors. *In vitro* exposure of BT-474 cells to 0.1 to 1 μM concentration 1α(OH)D_5 showed significant antiproliferative action compared to the vehicle-treated group. A similar effect was observed with 1,25 (OH)D3 at 10 times lower concentration. 1α(OH)D_5 induced cell differentiation, as evident from increased accumulation of lipid droplets in treated cells compared to that observed in control vehicle treated cells.

Previously, we have shown that 1α(OH)D_5 inhibits the *in vivo* and *in vitro* growth of the UISO-BCA-4 human breast carcinoma cell line.[12,13] The latter cell line is established in our own laboratory. UISO-BCA-4 cells are well differentiated and grow at an extremely slow rate when transplanted into athymic mice.[13] This study was performed in two commercially available established cell lines (BT-474 and ZR-75-1). 1α(OH)D_5 supplemented in the diet significantly inhibits the *in vivo* growth of both these cell lines transplanted in athymic mice. Histopathological examination of the tumors in the control and treated groups suggests that 1α(OH)D_5 supplemented in the diet influences the phenotypic characteristics of the breast cancer cells. Changes observed in the tumor cell size, nuclear morphology, and growth pattern of the cells in the stroma of tumors developed in animals receiving dietary supplement of 1α(OH)D_5 suggest that 1α(OH)D_5 induces phenotypic changes which render them less aggressive and thus inhibit their proliferation. Recently, EB-1089, a synthetic nontoxic vitamin D analog, has been shown to inhibit growth of LnCap prostate cancer cells transplanted into athymic mice.[42] The histopathology of the xenograft tumor obtained from animals receiving s.c. administration of EB-1089 was similar to that observed in our study.[42] Our results on 1α(OH)D_5 and those reported for the EB-1089 vitamin D analog suggest that vitamin D-like compounds could have potential therapeutic value in various different cancer types.

Detailed study performed in the ZR-75-1 xenograft model revealed an important finding. Even though dietary treatment with 1α(OH)D_5 for 45 days showed significant growth inhibitory action, the time of treatment received was not sufficient to irreversibly inhibit the growth of these tumors, as discontinuation of dietary supplement of 1α(OH)D_5 restored the tumor growth to that in the control group. Histopathological examination of the tumor on day 45 explains why the tumor continued to grow after discontinuation of 1α(OH)D_5 supplement. Even though

1α(OH)D_5 treatment reduced the number of cells present in the tumor and most of the cells showed characteristic phenotypic changes, the presence of a few cells similar to those observed in the control group was evident. This further explains why the histology of the tumor following discontinuation of 1α(OH)D_5 treatment is similar to that in the control group. These results suggest that either 45 days treatment with dietary 1α(OH)D_5 supplement is not sufficient to influence all the tumor cells or some cells are resistant to 1α(OH)D_5 treatment.

We also determined the antiproliferative effect of Her-2 antibody. In our study, Her-2 antibody treatment alone was not effective at reducing proliferation of BT-474 cells. Recently, Lane *et al.*[33] studied the effect of Her-2 antibody on *in vitro* proliferation of BT-474 cells and showed that Her-2 antibody significantly reduces the growth of BT-474 cells. This latter discrepancy in our results and that reported by Lane *et al.* could be attributed to the difference in the antibody concentration used, in the type of antibody used, or in the source of antibody obtained. In our study, we used 1.5 μg/ml Her-2 antibody obtained from Neomarkers, whereas Lane *et al.* used 4D5 antibody at 10 μg/ml concentration obtained from Genentech Inc., San Francisco, CA. When cells were exposed to both Her-2 antibody (1.5 μg/ml) and 1α(OH)D_5 simultaneously, significantly higher growth inhibitory action was noticed than with either compound given alone.

We further studied *in vivo* whether combined treatment with 1α(OH)D_5 and Her-2 antibody would be better than either agent given alone. Our study clearly indicated that combination treatment was more effective in not only inhibiting the growth of tumors but also maintaining tumor growth rate at a base line level. Histological examinations of tumor suggest that tumor cells have lost their ability to divide, and they remain as small clusters embedded in the stroma. Further withdrawal studies performed in ZR-75-1 cells suggest that, even after

cessation of treatments, tumors in animals receiving combination treatment fail to grow. These results lead us to believe that Her-2 antibody and 1α(OH)D_5 both have different independent actions on breast cancer cells and therefore treatment with 1α(OH)D_5 would enhance the efficacy of Her-2 antibody given at even a very low concentration.

Generally 1,25 (OH)2 vitamin D_3 is limited in clinical applicability in spite of its cell-differentiating and antiproliferative actions due to its toxicity.[14,15,16,17] 1α(OH)D_5 supplemented in the diet with or without Her-2 antibody showed no major deleterious effects, and appears to be relatively non toxic. These results further support our previous finding in rats.[18] In animals bearing ZR-75-1 or BT-474 xenografts, actual body weight during the course 1α(OH)D_5 was less than that in the control group receiving regular diet. However, one week after cessation of diet, body weight was significantly different in all groups. Also, dietary supplementation of 1α(OH)D_5 did not influence regular mobility or appetite of animals, suggesting no apparent effect in this group. Also, determination of serum calcium levels failed to show hypercalcemia in animals receiving only 1α(OH)D_5 in the diet. Higher serum calcium levels were observed only after combination treatment with Her-2 antibody and 1α(OH)D_5; however, the latter effect was reversible, and normal calcium levels were observed in animals after discontinuation of treatment. We believe that, in combination treatment, the higher calcium levels that were observed in mice may be unrelated to 1α(OH)D_5 supplement and could be a cumulative effect of the antibody, estradiol, and treatments on calcium-binding proteins, resulting in increased calcium levels.[14] These results strongly suggest that 1α(OH)D_5 is a relatively nontoxic vitamin D analog with potent growth-inhibitory effect in athymic mice, and the analog in combination with Her-2 antibody may offer a novel highly effective therapeutic option, especially for those patients with tumors overexpressing Her-2 receptors and undergoing Her-2 antibody therapy.

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Figure Legends

Figure 1 Effect of 1α(OH)D_5, Her-2 antibody, and 1α(OH)D_5 and Her-2 antibody combination treatments on *in vitro* growth of BT-474 cells. The cells were exposed to indicated concentrations of 1α(OH)D_5 and Her-2 antibody alone or in combination for 7 days. Data represent % of cells in relation to vehicle-treated control. Mean value obtained in the vehicle-treated group was considered as 100%. Data represent mean + SE of 4 observations. *indicates $p<0.05$ (compared to control).

Figure 2 Effect of 1α(OH)D_5, Her-2 antibody, and combination of both on morphology of BT-474 cells. The cells were exposed *in vitro* for 7 days to a) Vehicle only; b) 0.1 μM concentration of 1α(OH)D_5; c) 0.1 μm concentration of 1,25$(OH)_2D_3$; d) Her-2 antibody at 1.5 μg/ml concentration; e) Her-2 1.5 μg + 10^{-7} M 1α(OH)D_5. Arrow shows presence of intracytoplasmic vacuoles.

Figure 3 Staining for intracytoplasmic lipid droplets in a)control vehicle; b) 10^{-7} M 1α(OH)D_5; c) Her-2 antibody; d) Her-2 antibody + 1α(OH)D_5-treated cells. Arrow shows lipid droplets.

Figure 4 Effect of 1α(OH)D_5 supplemented in the diet, Her-2 administration, and combination treatment with dietary 1α(OH)D_5 and Her-2 administration on *in vivo* growth of BT-474 cells transplanted in female athymic mice. Data represent mean ± SE value.

Figure 5 Histology of the BT-474 tumors originated in athymic mice receiving a) control diet; b) diet supplemented with 1α(OH)D_5 (12.5 μg/kg diet); c) control diet +

Her-2 antibody(5 μg/animal); d) 1α(OH)D_5-supplemented diet + Her-2 antibody. All groups received specified diet until termination of the experiment.

Figure 6 Effect of 1α(OH)D_5 supplemented in the diet, Her-2 administration, and combination treatment with dietary 1α(OH)D_5 and Her-2 administration on *in vivo* growth of ZR-75-1 cells transplanted in female athymic mice. Data represent mean ± SE value.

Figure 7 Histology of ZR-75-1 xenografts on day 45 from animals receiving a) control; b) 1α(OH)D_5-supplemented diet.

Figure 8 Growth of ZR-75-1 xenografts after discontinuation of 1α(OH)D_5 dietary supplementation and Her-2 antibody administration as described in Figure 7. Data represent mean ± SE value.

Figure 9 Histopathology of ZR-75-1 xenografts at the time of termination. Animals received a) control diet; b) diet supplemented with 1α(OH)D_5; c) Her-2 antibody and control diet; d) Her-2 antibody + dietary supplement of 1α(OH)D_5. All animals received specified treatments for 45 days, and then all animals were switched to regular control diet. No group received Her-2 antibody therapy after 45 days. Growth of the xenograft was monitored until day 64. Animals were sacrificed, and tumor tissues were processed for histopathological observation.

Figure 10 Serum calcium levels in animals receiving control diet, 1α(OH)D_5-supplemented diet, Her-2 antibody, and Her-2 antibody + dietary 1α(OH)D_5 supplement. Data represent mean ± SE value. *indicates $p<0.05$ (compared to control).

Figure 11 Body weight (% of control) in ZR-75-1-bearing animals receiving control diet, 1α(OH)D_5-supplemented diet, Her-2 antibody treatment, and a combination of

Her-2 antibody and 1α(OH)D_5 treatment. Body weights are shown at the time of termination of experiment (45 days after treatment). Recovery in body weight was determined 1 week after discontinuation of 1α(OH)D_5 and Her-2 antibody treatment. * indicates $p<0.05$ (compared to control).

Figure 12 Serum calcium levels in animals bearing ZR-75-1 xenogrft after 45 days treatments as indicated and three weeks after discontinuation of treatment. Data represent mean ± SE value. * indicates $p<0.05$ (compared to control).

FIGURE 1

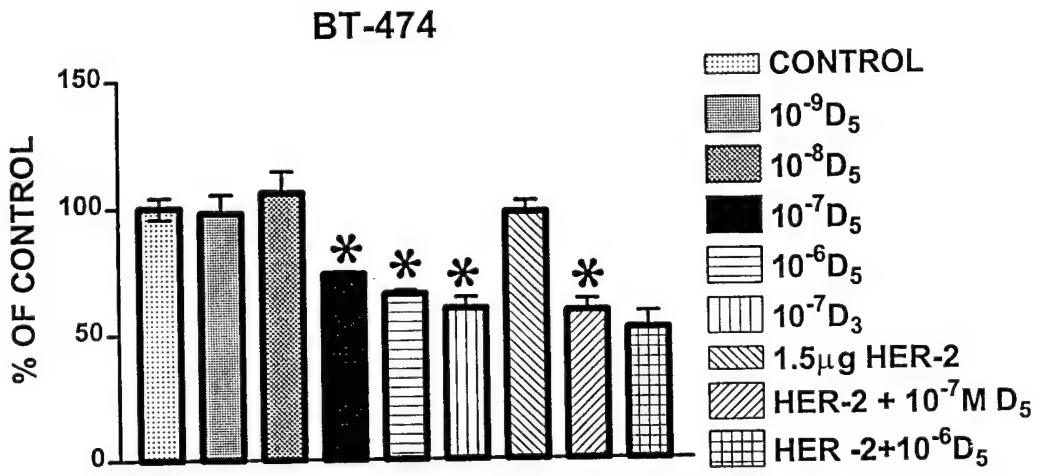

BT-474
% OF CONTROL
150
100
50
0
*
*
*
*
CONTROL
10-9D5
10-8D5
10-7D5
10-6D5
10-7D3
1.5µg HER-2
HER-2 + 10-7M D5
HER -2+10-6D5

FIGURE 2

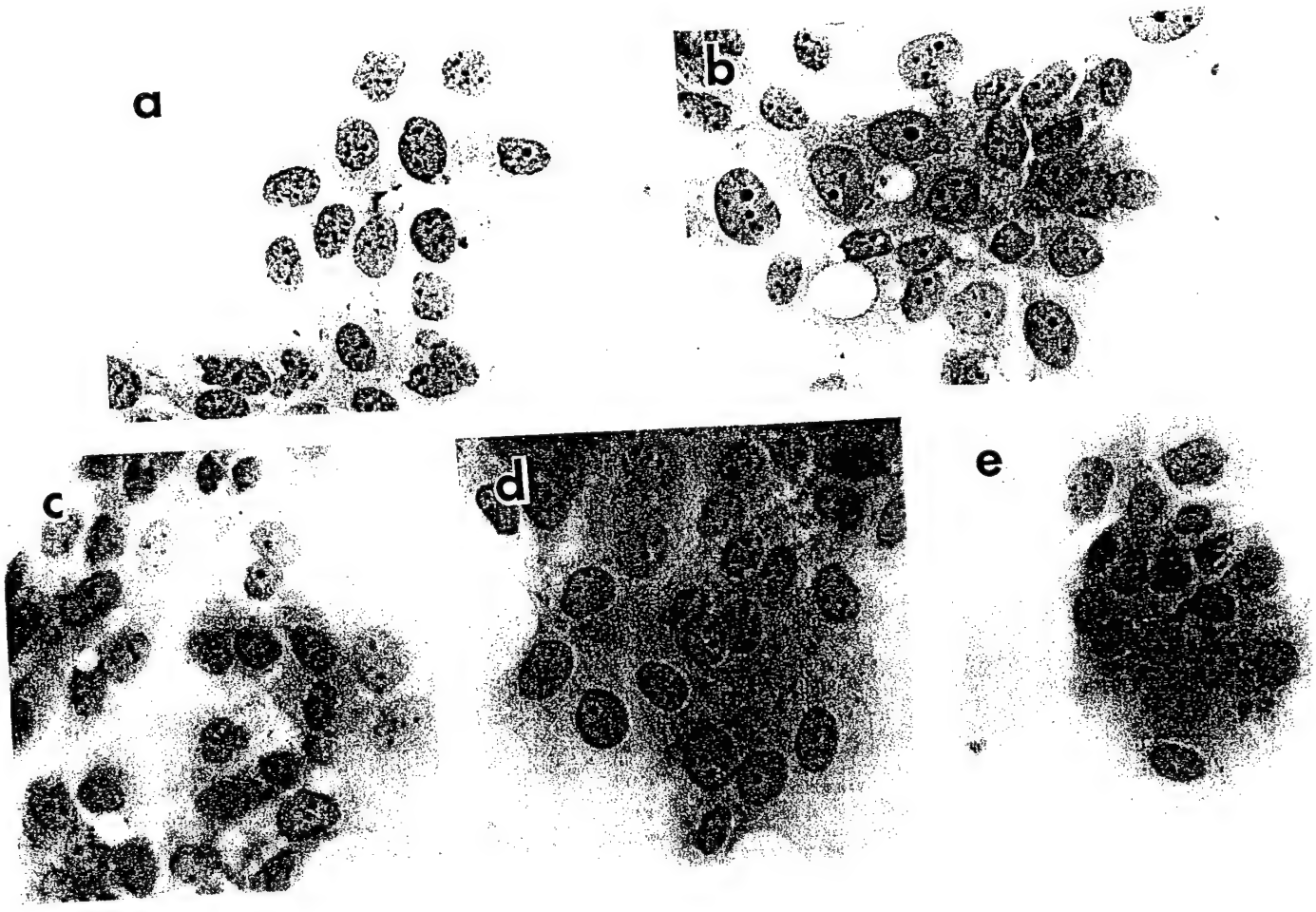


FIGURE 3

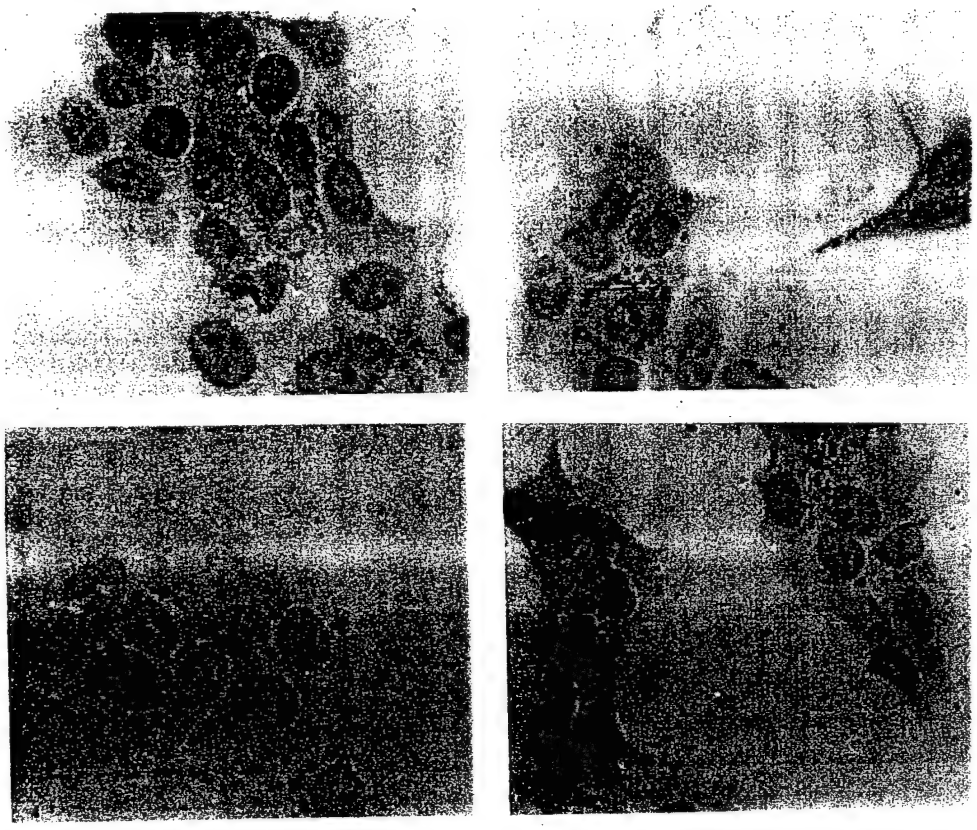


FIGURE 4

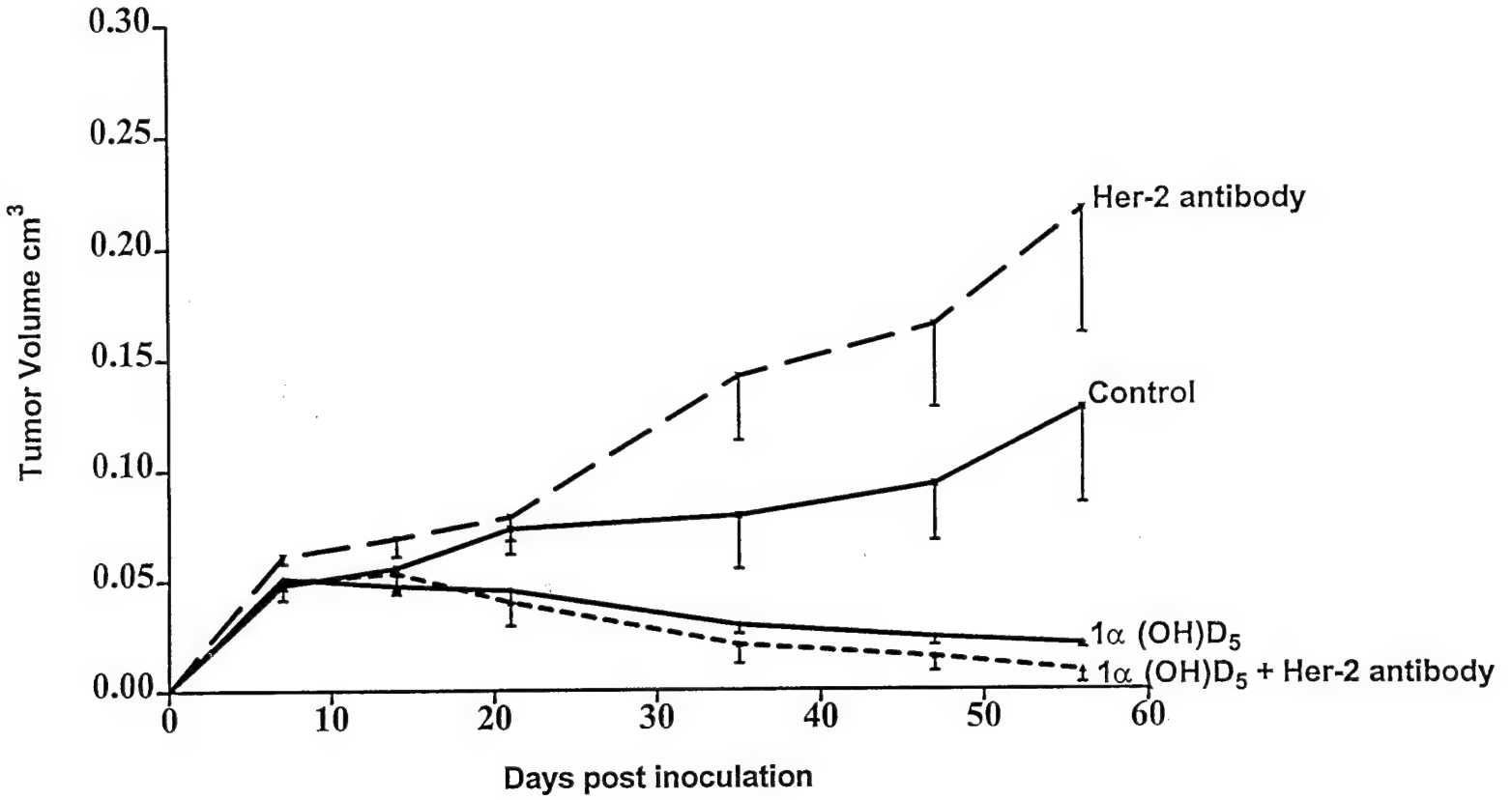

0.30
0.25
0.20
0.15
0.10
0.05
0.00
Tumor Volume cm3
0
10
20
30
40
50
60
Days post inoculation
Her-2 antibody
Control
1α (OH)D5
1α (OH)D5 + Her-2 antibody

FIGURE 5

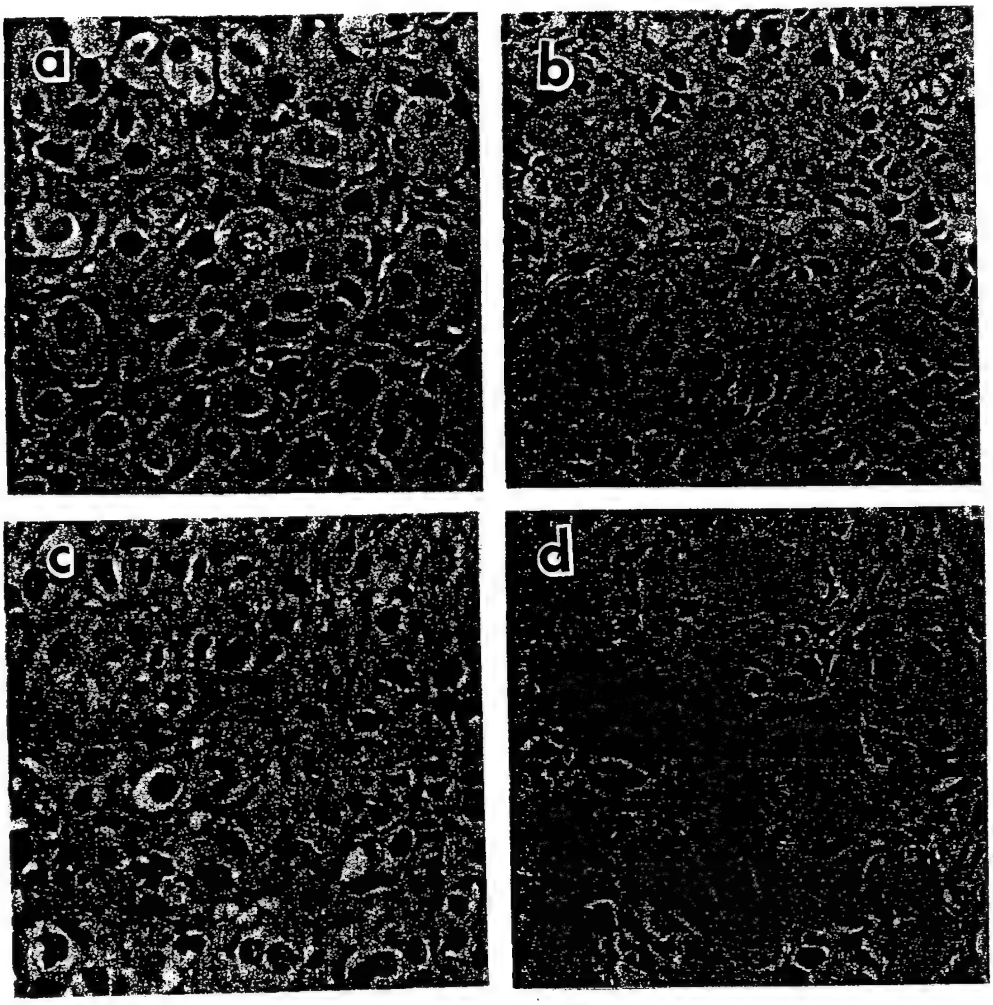


FIGURE 6

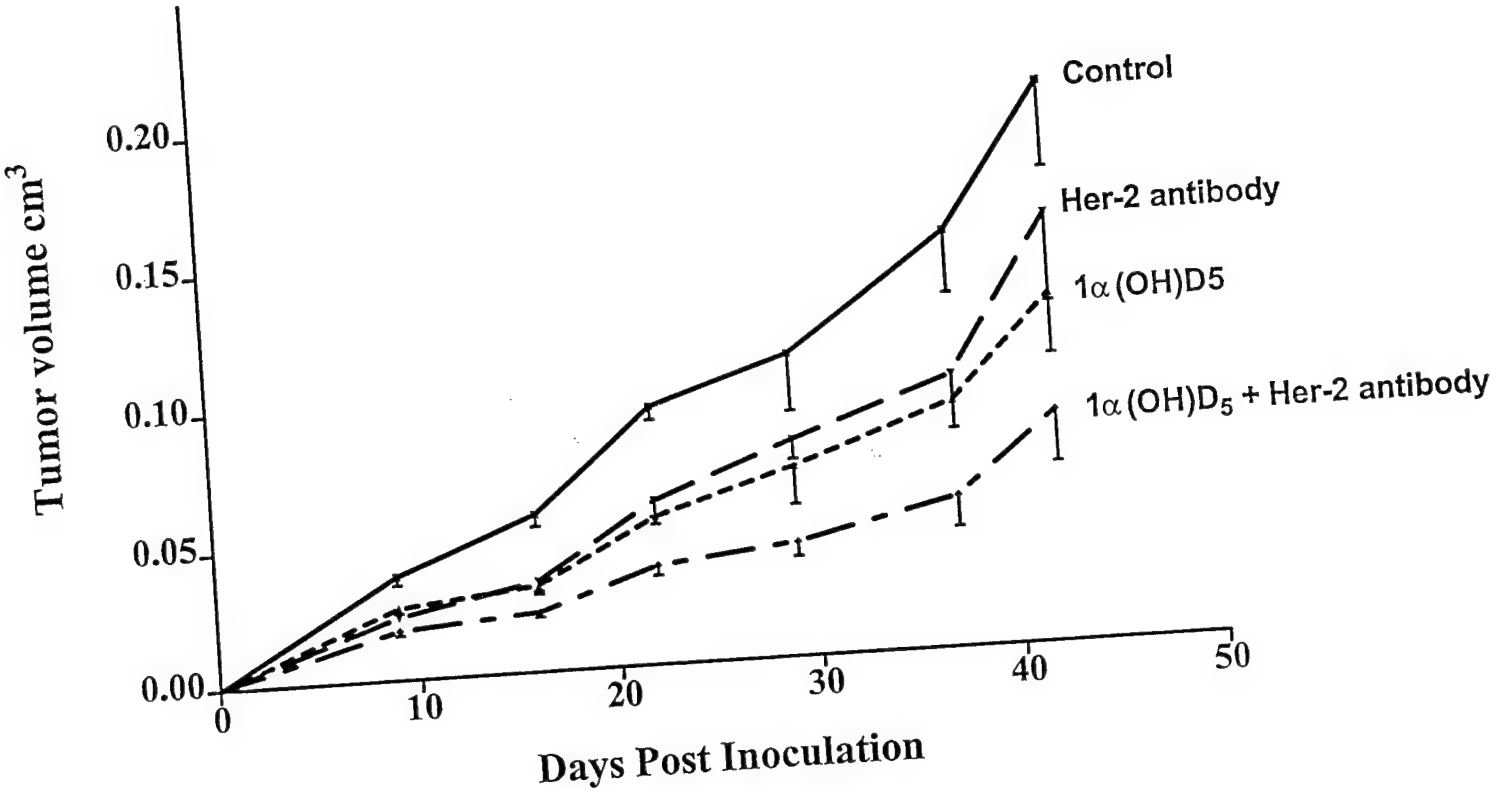

Tumor volume cm3
0.20
0.15
0.10
0.05
0.00
0
10
20
30
40
50
Days Post Inoculation
Control
Her-2 antibody
1α(OH)D5
1α(OH)D5 + Her-2 antibody

FIGURE 7

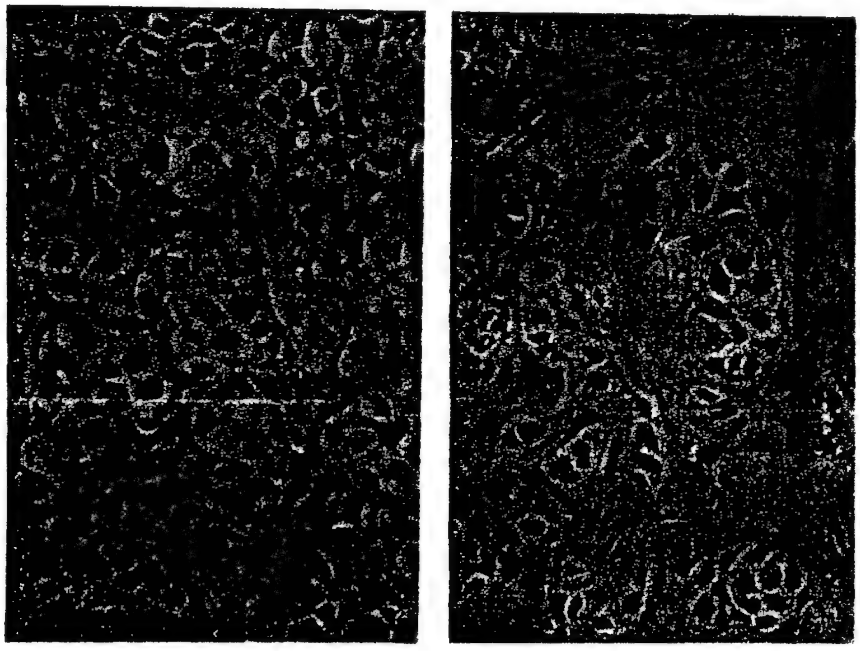


FIGURE 8

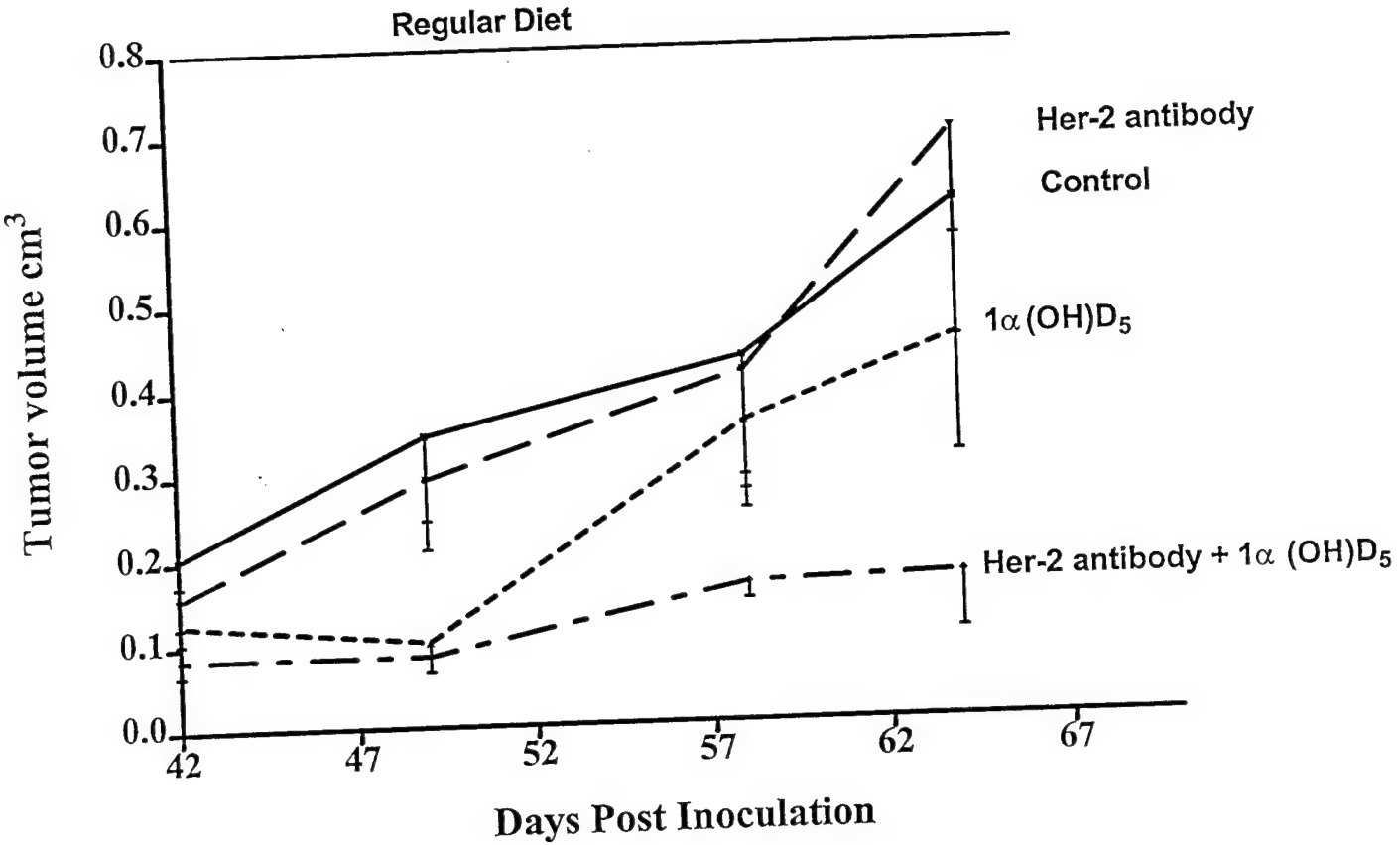

Regular Diet
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Tumor volume cm3
Her-2 antibody
Control
1α (OH)D5
Her-2 antibody + 1α (OH)D5
42
47
52
57
62
67
Days Post Inoculation

FIGURE 9

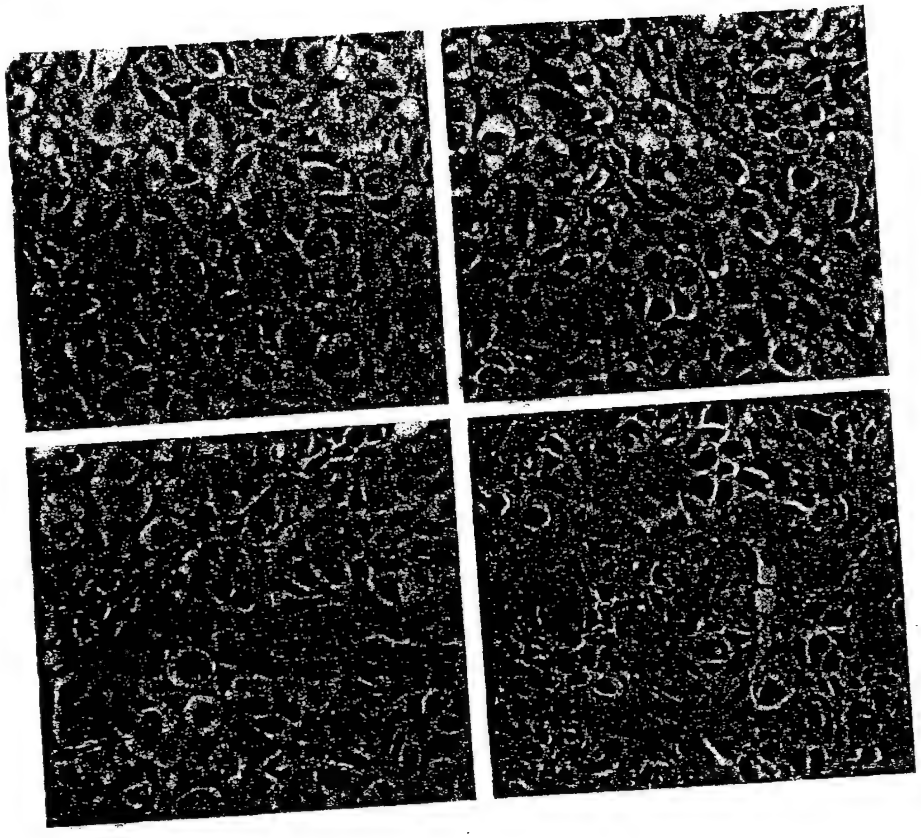


FIGURE 10

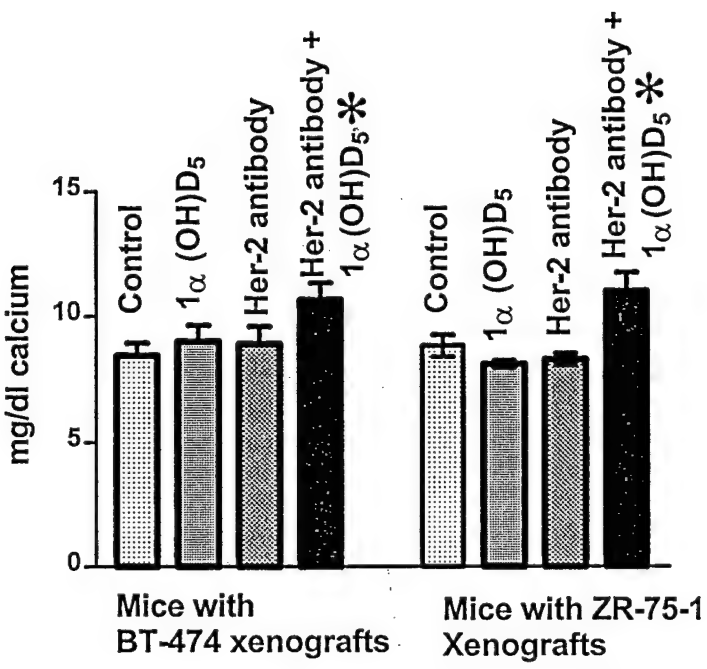

mg/dl calcium
15
10
5
0
Control
1α (OH)D5
Her-2 antibody
Her-2 antibody + 1α(OH)D5 *
Control
1α (OH)D5
Her-2 antibody
Her-2 antibody + 1α(OH)D5 *
Mice with BT-474 xenografts
Mice with ZR-75-1 Xenografts

FIGURE 11

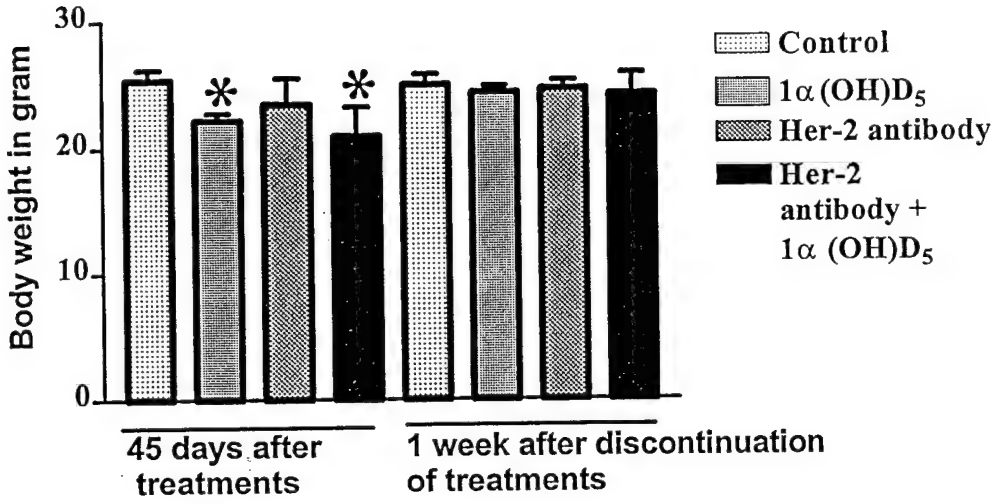
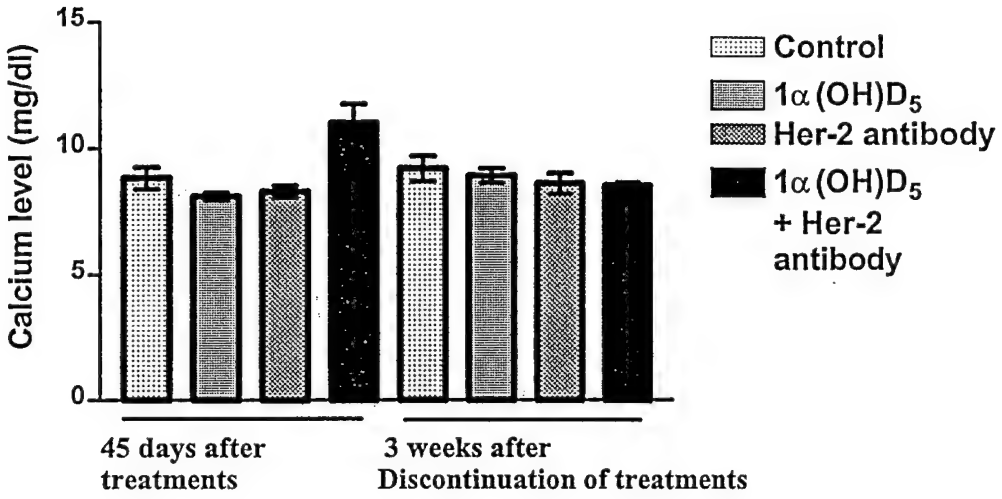

30
20
10
0
Body weight in gram
*
*
Control
1α (OH)D5
Her-2 antibody
Her-2 antibody + 1α (OH)D5
45 days after treatments
1 week after discontinuation of treatments

FIGURE 12


15
10
5
0
Calcium level (mg/dl)
Control
1α(OH)D5
Her-2 antibody
1α(OH)D5 + Her-2 antibody
45 days after treatments
3 weeks after Discontinuation of treatments

#4287 **Induction of α2-integrins during differentiation of breast cancer cells by 1α(OH)D_5.** Mehta R.R., Graves J.M. and Mehta R.G. *Department of Surgical Oncology, University of Illinois, Chicago, Illinois 60612.*

We have previously shown that 1α(OH)D_5, a non-calcemic synthetic analog of vitamin D inhibits cell growth and induce cell differentiation in various ER− and ER+ breast cancer cell lines. The cells pretreated with 1α(OH)D_5 for 7–10 days failed to form tumors when transplanted into athymic mice. Integrins, especially α2-antegrin has been shown associated with differentiation of human breast cancer cells. Transfection of breast cancer cells with vector containing α2 gene induces cell differentiation and fails to induce tumors in nude mice. We evaluated alteration in α2 integrin (m-RNA, protein level) in UISO-BCA-4 cells following 1α(OH)D_5 treatment. Immunohistochemically, a dose dependent increase in α2-integrin expression was observed following 7 days treatment with 1α(OH)D_5 (10^{-9} to 10^{-6} M). At 10^{-6} M 1α (OH) D5 treatment, 160% increase in α2 integrin was evident by FACS analysis. RT-PCR analysis using α2-integrin specific primers indicated that 1α(OH)D_5 causes increase in the expression of α2 m-RNA by 180% and 512% compared to controls at 24 hours and 48 hours respectively. The α-2 induction is declined to a control level by 7 days. Our results suggest that 1α(OH)D_5 is a modulator of molecular markers associated with breast cancer cell differentiation. (Supported in part by US Army grant DAMD-17-97-7263)

#4288 **Suppression of estrogen inducible growth by 1α-hydroxyvitamin D_5 is mediated by vitamin D receptors in human breast cancer cells.** Rothschild L.G., Kansagra A.M., Graves J., Mehta R.R., and Mehta R.G. *Department of Surgical Oncology, University of Illinois, Chicago, IL 60612.*

Although the role of vitamin D and its analogs as a differentiation agent for breast cancer cells is well established, the toxicity associated with active metabolite of vitamin D has precluded its clinical application. Earlier, we reported that 1α hydroxyvitamin D_5, (D_5) is a new non-calcemic analog, which induces differentiation of both ER+ and EF− breast cancer cells. In the present study we evaluated effects of D_5 on estrogen inducible growth of ZR-75-1 ER+ breast cancer cells. Results showed that only the cells treated with 10^{-9}M estradiol were growth inhibited by 5×10^{-7}M D_5 by 75% after 7 days in culture. Cells incubated with stripped medium were unaffected. Effects of D_5 on the induction of vitamin D receptor (VDR), TGFβ and TGFβ-R in these cells was determined by RT-PCR during a three-day period. Results showed that VDR induction by D_5 peaked at 24 hours after the D_5 treatment. On the other hand, the induction of p21 mRNA increased subsequent to the induction of VDR. There was no correlation among the expressions of VDR, TGFβ and TGFβ-R. Results suggest VDR mediated induction of p21 may be necessary in the suppression of estrogen inducible growth by D_5. (Supported in part by US Army grant DAMD-17-97-7263)

ENDOCRINOLOGY/PRECLINICAL AND CLINICAL 2/ PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS 7: Vitamin D, Retinoids, and Differentiation

#702 Modulation of cell surface proteins by 1a(OH)D5: Induction of breast carcinoma cell differentiation. Mehta, R.R., Graves, J.M. *Dept. of Surgical Oncology, University of Illinois at Chicago, Chicago, IL 60612 USA.*

Vitamin D and its analogues have been shown to have antiproliferative and cell-differentiating action in breast carcinoma cells. Recently, we studied the effect of a new synthetic, non-calcemic vitamin D analog $1\alpha(OH)D_5$ on human breast carcinoma cells. When human breast carcinoma cells (UISO-BCA-4, ZR-75-1) were treated in culture with 0.1–1 μM concentration of $1\alpha(OH)D_5$, it induced morphological alterations such as formation of numerous vacuoles and thinning of cytoplasm. It also enhanced the expression of casein and ICAM-1. We also observed increased accumulation of lipid droplets in cells treated with $1\alpha(OH)D_5$ than in those treated with vehicle only. In the present study, we further evaluated whether morphological changes in breast carcinoma cells after $1\alpha(OH)D_5$ treatment are due to altered expression of various cell surface proteins such as integrins (α2, α3, α5, α6, β1, β4) and cytokeratins (CK-8, CK-18, CK-19). After $1\alpha(OH)D_5$ treatment, we observed increased expression of cell surface alpha2 integrin and CK-18 in UISO-BCA-4 breast carcinoma cells. No significant changes were noticed in the expression of other biomarkers studied. Our results suggest that $1\alpha(OH)D_5$ has potent cell-differentiating action on malignant breast cells. This non-calcemic analogue could be of great therapeutic value in breast cancer treatment. (Supported in part by Department of Army, #DAMD17-97-1-7263.)

#1329 **Evaluation of toxicological parameters for 1α-hydroxyvitamin D_5, a novel analog of vitamin D.** Mehta, R.R., Christov, K., Hawthorne, M., Graves, J., Parikh, K. and Mehta, R.G. *Department of Surgical Oncology, University of Illinois, Chicago, IL 60612.*

The clinical use of vitamin D as an antiproliferative and differentiating agent has been precluded due to toxicity associated with it. Recently we synthesized an analog of vitamin D, 1α-hydroxyvitamin D_5 (D_5), and reported its chemopreventive activity. In the present study, we measured the MTD for D_5 and compared with 1α,25 dihydroxy D_3 (D_3), the active metabolite of vitamin D_3. Results showed that rats fed D_5 did not exhibit any loss of body weight gain up to 100 μg/kg diet as compared to 12.5 μg/kg diet of D_3. Similarly, D_5 can be tolerated at 200 ng concentration compared to 10 ng D_3 when injected i.p. three times a week for 2 months. The calcium concentration was not increased in the D_5 treated rats and mice however, plasma concentration of Phosphorous was elevated. Histopathological evaluations showed that there was no toxic effect of D_5 on kidneys, liver, mammary glands, or muscle. HPLC analysis of tissue extracts exhibited two major metabolites, one corresponded to dihydroxyvitamin D_5 and the other less polar metabolite remains to be identified. These results suggest that 1α-hydroxyvitamin D_5 is a relatively non-toxic analog of vitamin D and can be developed as a chemopreventive agent and/or as an adjuvant to chemotherapeutic agents.

#1545 **TARGETING HUMAN BREAST CARCINOMA CELLS WITH $1\alpha(OH)D_5$ LINKED TO HER-2 ANTIBODY.** Rajeshwari R Mehta, Jewell Graves, and Tapas K Das Gupta, *Univ of Illinois Med Ctr, Chicago, IL, and Univ of Illinois Med Ctr at Chicago, Chicago, IL*

Previously we showed that $1\alpha(OH)D_5$, a novel nontoxic vitamin D analog, inhibits cell proliferation and induces markers associated with cell differentiation in human breast carcinoma cells. $1\alpha(OH)D_5$ treatment in vitro enhanced the expression of intracellular casein, cell surface integrin $\alpha 2$, and intracytoplamic lipid droplets. All human breast carcinoma cell lines expressing VDR, irrespective of ER, p53, and Her-2 status, showed cell-differentiating response to $1\alpha(OH)D_5$. In vivo in the athymic mice xenograft model, $1\alpha(OH)D_5$ supplemented in diet reduced in vivo growth of breast cancer. Herein, we aimed to further enhance the effect of $1\alpha(OH)D_5$ by specifically targeting human breast cancer cells. In vitro,

BREAST TUMOR TARGETED NOVEL THERAPY

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Breast cancer treatment is mainly limited to endocrine/cytotoxic chemotherapies, but these have serious toxic effects and have not resulted in sustained remission or cure. Most human breast carcinomas overexpressing Her-2 oncoprotein(s) do not respond to standard chemotherapy. Thus, we need to identify agents significantly valuable in treating highly aggressive breast cancer. In this study, we aimed to target breast cancer cells using a novel synthetic analog: 1α(OH)D5 conjugated to Her-2 antibody. 1α(OH)D5 is a vitamin D analog; it is non-toxic and has both growth inhibitory and specifically cell-differentiating actions on breast cancer cells. In vitro, breast cancer cells (MCF-7, ZR-75-1, UISO-BCA-4, and BT-474) treated with 1α(OH)D5 showed induction of intracytoplasmic casein, ICAM-1, and alpha2 integrin. They also showed enhanced accumulation of lipid droplets. After being exposed in vitro to 1α(OH)D5, breast cancer cells lose their in vivo tumorigenic potential. In vivo in athymic mice, 1α(OH)D5 supplemented in the diet inhibited the growth of human breast carcinoma cells ZR-75-1, BT-474, MCF-7, and UISO-BCA-4 without causing hypercalcemia. Similarly, Her-2 antibody (reactive to external domain of Her-2 receptor) alone at low concentrations had weak inhibitory effect on breast cancer cell growth in vivo and in vitro. Combined treatment with 1α(OH)D5 and Her-2 antibody showed significant growth inhibition on various breast cancer cell lines injected into athymic mice. We aimed to further potentiate the cell-differentiating property of 1α(OH)D5 by covalently linking it to Her-2 antibody. This compound could be specifically targeted to cancer cells, as normal cells express low levels of Her-2 but tumor cells (especially aggressive) frequently express high levels. 1α(OH)D5 was immunoconjugated to Her-2 antibody using SANPAH (N-Succinimidyl-6-[4'-azido-2'-nitrophenylamino]hexanoate) (20:1-SANPAH: antibody) as a linker. Her-2 antibody linked to SANPAH was conjugated to 1α(OH)D5 (20:1, 1α(OH)D5:Her-2 antibody) using photoreaction. The 1α(OH)D5-Her-2 conjugate was characterized for its electrophoretic mobility, binding to various breast cancer cells, and in vivo biodistribution. 1α(OH)D5-Her-2 conjugate showed ability to bind to Her-2 expressing cells and competed with Her-2 antibody (unconjugated) for Her-2 binding sites. Biodistribution study in athymic mice bearing breast tumors show that both unconjugated antibody and 1α(OH)D5-Her-2 conjugate accumulate mainly in tumors expressing Her-2 receptors. Whether 1α(OH)D5-Her-2 conjugate inhibits the growth of breast tumors in athymic mice is currently under investigation.

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